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Methods for evaluation of radioimmunoassay kits the selection of a commercial radioimmunoassay procedure for human placental lactogen.

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METHODS FOR EVALUATION OF RADIOIMMUNOASSAY KITS: THE SELECTION OF A COMMERCIAL RADIOIMMUNOASSAY PROCEDURE FOR HUMAN PLACENTAL LACTOGEN

by

Karen A. Wurtsmith

A Major Clinical Chemistry Critique
Submitted to the Faculty of Graduate Studies
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ABSTRACT

METHODS FOR EVALUATION OF RADIOIMMUNOASSAY KITS:
THE SELECTION OF A COMMERCIAL RADIOIMMUNOASSAY PROCEDURE
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Since most clinical laboratories do not have the time nor equipment to develop their own RIA (radioimmunoassay) procedures, they must rely on commercial manufacturers to supply them with the appropriate reagents--usually in the form of kits. Before being put into routine use, these kits should be evaluated for their sensitivity, accuracy, precision, specificity, and overall practicality.

Several tests which can be performed allow the clinical chemist to determine the quality of the reagents and the assay results. Tracer immunoreactivity studies may indicate a damaged tracer, while Scatchard plots give a general idea about antiserum affinity and heterogeneity. Parallelism studies are necessary to validate similarity of standards and unknowns. Accuracy of the assay may be confirmed by either recovery studies or comparison of results against a "reference" procedure. Sensitivity is often an important quality to evaluate if analyte is present in low concentrations; and precision must

also be determined to assess the reliability of the results. Practical aspects of the procedure must likewise be considered including such factors as: turn-around time; ease of performance; equipment necessary; and cost.

This paper discusses the qualities that are desirable in an assay, and then describes procedures used to evaluate these qualities. Application of these procedures is made to the selection of a commercial RIA kit for the quantification of HPL in maternal serum.

DEDICATION

To Jerry,

my dearest husband

ACKNOWLEDGEMENTS

I would like to particularly thank Dr. Carolyn Feldkamp for the vast amount of help she has given me. She patiently tolerated my unending questions, supplied me with numerous references, and ultimately, made me realize how interesting the field of radioimmunoassay really is. I would also like to thank the techs at the ligand assay lab of Henry Ford Hospital for being such nice people to work with. They made me feel at home, and were always willing to show or help me.

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Thanks also to the rest of my committee, Dr. Bennie Zak, and Dr. Norman Taylor; to Mary Kosinski from the special chemistry department of Hutzel Hospital, for supplying me with controls and patient samples to run in split-sample comparisons; and to the Endocrinology lab of HFH, for allowing me to use their equipment for chromatography, and for supplying me with hGH and HCG solutions.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ab	antibody
Ag	antigen or ligand
AgAb	antigen-antibody complex
Ag^*	labeled antigen
B	amount of radioactivity bound by antibody
B_O	maximum binding, amount of radioactivity bound in the zero dose tube
b	slope of the dose response curve; y-intercept of a line
BSA	bovine serum albumin
B/F	ratio of bound to free labeled ligand
CPM	counts per minute
C.V.	coefficient of variation
DPM	disintegrations per minute
ED_x	estimated dose at x percent binding
E	counter efficiency
ϵ	relative experimental error (Ekins)
F	amount of free (unbound) radioactivity
λ	index of precision (s/b)
[H]	concentration of hormone or unlabeled ligand (Yalow)
hGH	human growth hormone
HCG	human chorionic gonadotropin
HPL	human placental lactogen
HSA	human serum albumin
K_{eq}	equilibrium constant
K	K_{eq} or equilibrium constant, usually pertaining to unlabeled ligand

K_{std}	K_{eq} of standards
K^*	K_{eq} of tracer
k_f	rate constant of the forward reaction
k_r	rate constant of the reverse reaction
LDD	least detectable dose
logit Y	ln(Y/(1-Y)
m	slope of the line (y = mx + b)
n	number of samples or number of replicates
NSB	non-specific binding ("blank" count)
NSS	normal sheep serum
p	unlabeled antigen or ligand
p^*	labeled antigen or ligand
$\Delta'p$	a change in the amount of unlabeled hormone (Ekins definition of sensitivity)
PBS	phosphate buffered saline
q	antibody or binder
r	conversion constant between CPM and μCi; correlation coefficient
R_o	ratio of free to bound activity at zero concentration (Ekins)
$\Delta'R$	change in the ratio of free to bound activity (Ekins)
RIA	radioimmunoassay
s	standard deviation
s^2	variance
σ	specific activity of tracer (curies/mass)
S	specific activity of tracer (Ekins)
S.A.	specific activity of tracer
SD_d	standard deviation of the differences

S_y	standard error in the estimate of Y
T	total count; counting time (Ekins)
V	volume of reaction mixture (Ekins)
Y	dose response variable
[ ]	molar concentration

CHAPTER I

INTRODUCTION

Radioimmunometric Concepts

Until the development of the technique of radioimmunoassay (RIA) or saturation analysis in the mid 1950's, measurement of many molecules present in minute quantities in biological fluids was not possible. In 1960 Solomon A. Berson and Rosalyn S. Yalow, working at the Veterans Administration Hospital in New York, published results which were the beginnings of a continued almost exponential growth of a now prominant and important tool of clinical chemistry. They developed a method for the assay of insulin using specific insulin antibodies (1). Another related assay method using a specific binding protein instead of antibody was, at the same time, being developed by Roger P. Ekins at the Middlesex Hospital Medical School in London (2). Ekins described a method for serum thyroxine assay using thyroxine binding globulin as a specific reagent in the system.

RIA offered several distinct advantages over conventional chemical or bioassay methods of that time. It provided greater sensitivity (usually several orders of magnitude), high specificity, and the possibility of performing simultaneous determinations on large numbers of specimens. RIA had special impact on the quantification of peptide hormones which, up until then, were in too small of quantities and of too similar structure

to measure accurately with available methods. Over the years, the use of radioimmunological methods has been extended to almost any type of molecule for which specific antibodies can be produced.

The basic principle of all saturation assay techniques (including RIA) was described in a novel way (3) using a simple model of a jug of water and a glass. Assuming that the jug has a larger capacity than the glass, if the entire contents of the jug were poured into the glass some of the water would overflow. The proportions of water remaining in the glass and that overflowing would depend only upon the total amount of water initially in the jug. In other words, the amount of water in the jug (ligand concentration) can be measured by using a limited and fixed capacity glass (antibody or binding agent) and determining the amount of water in the glass (bound ligand fraction) versus the amount of water spilled over (free ligand fraction).

In RIA and other radiometric techniques the two fractions can be measured by use of a radioactively labeled ligand. Competition occurs between the labeled and unlabeled ligand or antigen for a specific protein or antibody binding site that is in limited concentration. The unlabeled antigen (Ag) and antibody (Ab) form a soluble antigen-antibody complex (AgAb) by a reversible reaction:

$$Ag + Ab \underset{k_r}{\overset{k_f}{\rightleftharpoons}} AgAb \qquad \text{(Eq. 1)}$$

The kinetics of this reaction can be treated identically to enzymatic reactions and is governed by law of mass action.

Therefore, $$\text{rate}_f = k_f[Ag][Ab] \qquad \text{(Eq. 2)}$$

$$\text{rate}_r = k_r[AgAb] \qquad \text{(Eq. 3)}$$

where k_f and k_r are constants associated with the forward and reverse reaction, respectively. At equilibrium $\text{rate}_f = \text{rate}_r$ and substitution and rearrangement results in:

$$\frac{k_f}{k_r} = \frac{[AgAb]}{[Ag][Ab]} = K_{eq} \qquad \text{(Eq. 4)}$$

K_{eq} (or K) is the equilibrium constant which describes the ratio of bound product to free reactants at equilibrium, and which is dependent on the affinity of the antibody for the antigen. The equilibrium constant, in fact, is commonly used as a measure of this affinity.

Labeled antigen (Ag^*) undergoes a similar reaction:

$$Ag^* + Ab \rightleftharpoons Ag^*Ab \qquad \text{(Eq. 5)}$$

If unlabeled antigen is added to the above system (Eq. 5), a competition results between labeled and unlabeled ligands for antibody sites, which ultimately results in less binding of the labeled species. Higher concentrations of unlabeled or "cold" antigen cause less and less binding of "hot" antigen. If the ratio of bound to free labeled antigen (B^*/F^* or Ag^*/Ag^*Ab) is plotted versus cold ligand concentration a characteristic hyperbolic curve results (Fig. 1) that is typically called the dose response curve. Known standard concentration solutions of unlabeled antigen are run along with unknown specimens so that the B/F ratios** of unknowns can be

**Conventionally B^*/F^* is designated as B/F. It is assumed that we are looking at the ratio of the labeled antigen since this is the only species that can be measured.

FIGURE 1

THE TYPICAL DOSE RESPONSE CURVE

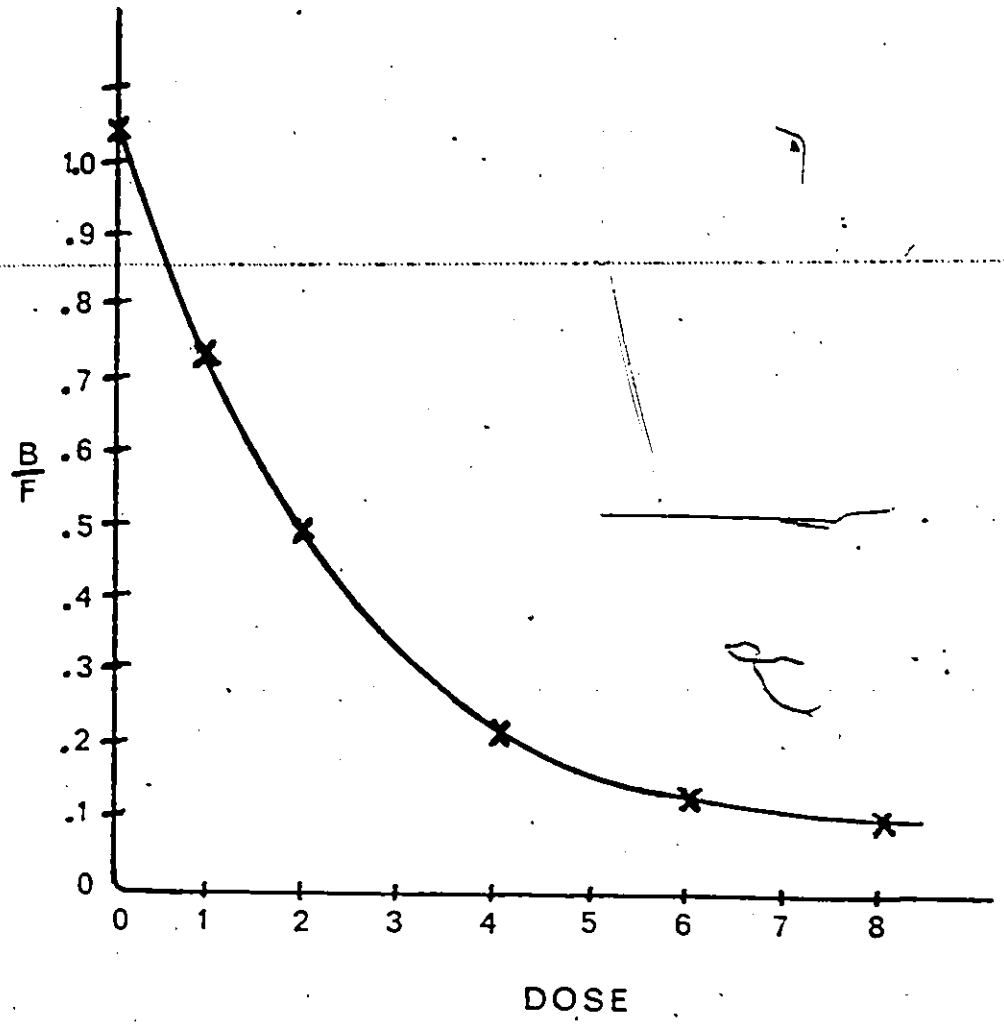
Legend

A plot of cold ligand concentration on the x-axis and the ratio of bound to free labeled ligand on the y-axis will yield the typical hyperbolic curve of competitive radioassay procedures.

Reference: Goldsmith, S. J.: "Radioimmunoassay: Review of Basic Principles", Seminars in Nuclear Medicine, 5 (2), (1975), p. 127.

FIGURE 1

THE TYPICAL DOSE RESPONSE CURVE



compared to the standard curve and their concentrations obtained.

In order to obtain B/F, bound and free fractions must be separated for individual quantification. Several methods have become available and will be discussed below. All are based on the differences in physical and chemical characteristics between the bound complexes (Ag^*Ab and AgAb) and free antigen (Ag and Ag^*).

One point which deserves mention is the fact that, although RIA methods are more sensitive and more specific than bioassay procedures, the two do not necessarily provide comparable results. This is due to the fact that RIA measures the immunoreactive qualities of a molecule while bioassay measures the biologically active properties of that same molecule. Since antibodies may be produced against several different sites on an antigenic substance and not necessarily to the whole intact molecule, inactive molecular fragments and polymers, precursor molecules, or molecules with similar but not identical structure may be quantified radioimmunometrically and not by bioassay. Similarly, certain antibodies may not detect biologically active substances since the immunoreactive site in question may have become altered or destroyed. A classic example of the dissimilarities between immunoreactivity and biological activity is the study done by Silverman and Yalow on PTH (4). Using several different antisera to study PTH levels in normal subjects, uremic patients with secondary hyperparathyroidism, and patients with parathyroid carcinoma, they discovered that immunoreactive PTH present in human serum was actually composed of four fractions: a

prohormone; active PTH; and two inactive fragments. Studies done on insulin (5), gastrin (6), and ACTH (7) have also indicated non-biologically active immunoreactive precursor molecules.

RIA Kit Evaluation

Since it is not practical for individual laboratories to purify their own antigens, prepare their own antisera, and set up optimal RIA procedures, it has become advantageous for several commercial manufacturers to mass produce different RIA "kits" which laboratories can purchase. Kit components produced by different companies for the determination of the same antigen (for example human placental lactogen) do not have the same reaction characteristics, quality, or concentration. Evaluation of these kits is necessary to select the one which best suits the needs of the individual laboratory in terms of diagnostic accuracy, reliability, economic feasability, and overall practicality. Aside from validation of the kit itself, evaluation of a kit also serves to assess in-house quality control and technology--factors which have a great affect on the reliability of results reported to the physician.

Historically, four parameters have been used to determine the reliability of a clinical chemistry procedure, i.e., sensitivity, precision or reproducibility, specificity, and accuracy (8,9). It is worthwhile to look at these parameters individually in order to fully understand the significance

of each, what factors affect them in RIA procedures, and the methods used for their determination.

(a) Sensitivity

Sensitivity has classically been described as the "minimum amount of substance in biological medium that can be determined with accuracy, precision, and specificity" (9). In RIA there has been controversy over the exact definition of the term, and several investigators have several different opinions.

Midgley has described sensitivity as "the smallest amount of unlabeled antigen that can be distinguished from no antigen" (10). Yalow has taken a more theoretical approach, and defines sensitivity in terms of the slope of the dose response curve, i.e., the rate of change of B/F with change in absolute concentration of hormone ($d(B/F)/d([H])$) (11), or the smallest measurable change in response variable with a small change in ligand concentration. Her mathematical analysis results in the conclusion that the sensitivity of an assay is limited by K, the equilibrium constant, and that the maximum obtainable sensitivity is equal to $-\frac{4}{27}K$. She concludes that as $[H] \rightarrow 0$, maximal sensitivity or maximal slope at zero dose is obtained if the antibody is diluted to give 33 1/3% binding (B/F = 1/2) of a minute quantity of tracer.

Ekins disputes this approach to defining sensitivity and instead defines it as: the quantity of unlabeled hormone ($\Delta' p$) that will change the distribution of radioactivity by an

amount $\Delta'R$ that is just equal to the standard deviation (ΔR_o) of the experimental determination of R_o, where R_o is the ratio of free to bound activity at zero concentration of hormone (12). He regards sensitivity essentially, then, as precision at zero dose. In his analysis he gives emphasis to both the slope of the response curve and to the variance of the dose response metameter (dose response variable, e.g., F/B) or to error in its measurement. If the only error that is included in the estimate of R_o is counting error then: $\Delta'p = 4\sqrt{2}/KSVT$, where $\Delta'p$ is the minimum detectable concentration of hormone, K is reaction energy or equilibrium constant, S equals specific activity of the tracer, V is volume of the reaction mixture, and T equals counting time. This equation indicates that he similarly believes that sensitivity is limited by K, but also that it depends on other factors. He concludes that sensitivity is maximum if binding at zero antigen concentration is 50% or $R_o = 1$. This is achieved when antibody concentration is 3/K and labeled antigen concentration is 4/K. If other experimental errors besides counting are considered, then the calculations must include the factor ϵ or relative experimental error in the measurement of R_o. Optimal antibody and labeled antigen concentrations must then likewise be adjusted. Ekins recommends use of computer optimization techniques to accomplish this.

It must be mentioned that these above conclusions, although slightly different, are both supported by basic assumptions which must be fulfilled; and that if these assumptions are

invalid, as in many real-life RIA procedures, the resulting theoretically maximal obtainable sensitivities are also invalid. Rodbard (13) has summarized these assumptions which are:

1) The antigen or ligand is present in homogeneous form, consisting of only one chemical species.
2) The antibody or binding protein is present in only one homogeneous chemical form.
3) Both antigen and antibody are univalent; that is, one antigen can react with one molecule of antibody, but no other combinations can occur.
4) No allosteric or cooperative effects are present. That is, the antigen and antibody react according to the first order mass action law (second order chemical kinetics).
5) Radioactively labeled and unlabeled antigen have the same physical-chemical properties (aside from the presence of label on the former).
6) The antigen and antibody react until equilibrium is reached.
7) Bound and free forms of the labeled antigen can be separated perfectly without perturbing equilibrium.
8) The ratio of bound to free antigen or the ratio of bound to total antigen can be measured perfectly.

It is actually the deviations of an assay from these theoretical assumptions that necessitate the validation

of an assay's reliability--not only in the area of sensitivity, but also in precision, accuracy, and specificity. It is also these deviations which make it necessary to determine optimal reagent concentrations, incubation times, and other assay variables for each individual RIA procedure.

Briefly, some factors which may affect the sensitivity of an assay include: the value of K, specific activity of the tracer, antibody concentration, tracer concentration, and any condition which influences the antigen-antibody reaction or avidity such as pH, ionic strength, temperature, and presence of anticoagulants or bacteriostatic agents. The above mentioned variables will be illustrated and discussed in more detail in the following sections.

Tests to assess the sensitivity or potential sensitivity of an assay include: 1) determination of the least detectable dose from precision of the zero tube; 2) assay of dilutions of the lowest standard; 3) calculation of K by use of a Scatchard plot; and 4) calculation of the slope of the dose response curve near zero dose. These methods will be described under their appropriate sections.

(b) Precision

Precision or reproducibility is another parameter which is important to consider when evaluating an assay. Midgley describes precision as "the extent to which a given set of measurements of the same sample agrees with the mean, i.e., the amount of variation in the estimation of unlabeled

hormone" (10). Conventionally precision has been evaluated as the coefficient of variation (C.V.) at any point on the dose response curve. The C.V. or error at any dose level depends on two factors: the variation in the measurement of the dose response variable (B/F, B/T, logit Y, etc.); and the slope of the dose response curve. The relationship of these parameters to the precision is illustrated in figure 2. The index of precision, λ, which is defined as the error in the dose respone variable, s, divided by the slope, b, describes the relationship mathematically: $\lambda = s/b$.

Factors which affect s are those which are often called "experimental errors" and include practical considerations such as pipetting, mixing, incubating, decanting, and other physical variables. Also included in this type of error are counting discrepancies. Since radioactive decay is random and follows a Poisson distribution where $s = \sqrt{\bar{x}}$ (here $\bar{x}$ is the mean count rate or activity), it follows that greater count accumulation produces lower error in the estimate of the actual count rate.

Slope of the dose response curve (b), on the other hand, depends on the "chemistry" of the assay and is affected by such factors as antibody and labeled antigen concentration, tracer specific activity, tracer damage, and K.

Practically speaking, precision of an assay is estimated by the measurement of several dose levels of analyte, in several different assay set-ups, and several times during each set-up. In this way the C.V. at each dose can be calcu-

FIGURE 2

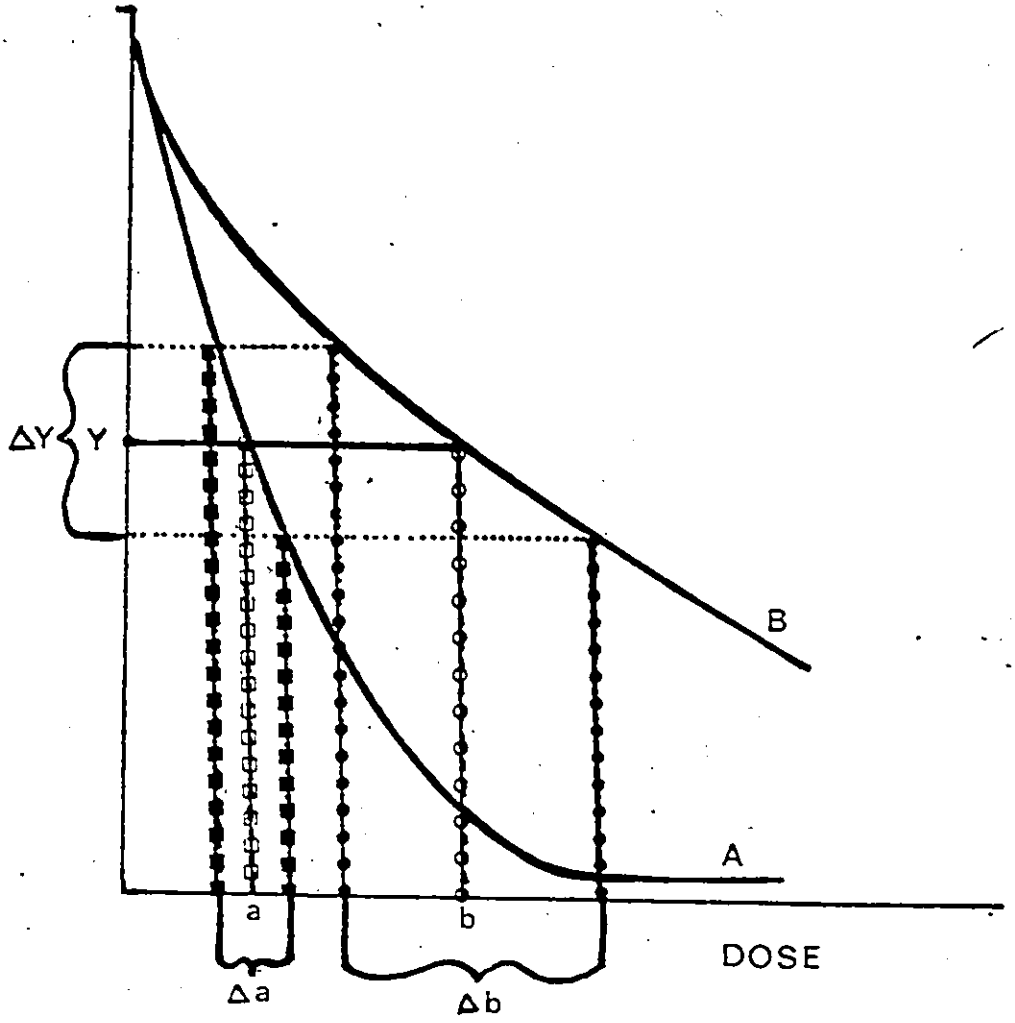
PRECISION OF AN ASSAY

Legend

Precision of an assay is dependent on both the variation in the measurement of the dose response variable (ΔY) and on the slope of the dose response curve. Mathematically this is stated as $\lambda = s/b$ where s is ΔY and b is the slope of the curve. Curve A has an initial slope which is large, and therefore Δa (variation on the dose axis or the imprecision) is small. Curve B with a shallower slope produces a large variation in b on the dose axis and thus the precision is poor. Also, an increase or decrease in ΔY would cause a corresponding change in Δa or Δb.

FIGURE 2

PRECISION OF AN ASSAY



lated as inter-assay and intra-assay variation. Another estimate of precision can be obtained by looking at the "estimated doses" at certain percent bindings on the standard curve. For example, the ED_{20} (estimated dose at 20% binding). ED_{50}, and ED_{80} for several runs can be accumulated and the C.V. calculated for each. This gives information only on the inter-assay variation. Precision of the assay is followed on a day-to-day basis by use of control samples with established values.

Another concept which accompanies the idea of precision is that of resolution. Resolution can be defined as the ability to distinguish between two analyte concentrations at any point on the standard curve. In order for one concentration value to be considered significantly different from a second, it must be outside of the 95% confidence interval of the second concentration point. Greater precision at any dose level will therefore result in a narrower confidence interval and greater resolution at that point.

One point that should be mentioned is that the method of data reduction may have an affect on the precision or variance of an assay at different dose levels. In particular, the logit-log transformation, because of factors inherent in the calculation of logit Y, shows striking heteroscedasticity at the low and high ends of the curve (10). This variability in estimation of the dose response metameter may cause increased variability at high and low hormone doses. Weighting functions should be used to minimize this effect.

(c) Specificity

Specificity can be defined as the freedom from interference by substances other than the one intended to be measured (10). In RIA, this specificity depends essentially on the singularity of the antiserum for binding of the desired antigen. If there are other compounds in the reaction mixutre which bind to the same site on the antibody as the tracer, they will cause a change in the B/F ratio that is not due to the desired antigen. These compounds could be structurally similar molecules, molecular fragments, precursor molecules, or polymers of the desired analyte. Additionally, if there is more than one binding site on the binder (antibodies have two) or there are multiple antibodies present (heterogeneous antiserum), and the antigen reacts with all of the sites but with different avidities, then the chance that there will be other cross-reacting substances in the mixture will be quite large. The fact that a heterogeneous antibody preparation is more the rule than the exception in RIA systems makes the total freedom from cross reactions almost impossible. Ekins deals with the theoretical implications of multiple competitors and multiple binding sites and the dose response curves that may result (12).

One method that has been used to attempt to eliminate cross reactivity is isolation of the selected antigen from the biological sample by extraction or purification. The disadvantage of extraction is that this procedure may produce artifactual errors by the introduction of chemical contaminants into the system. This may explain the positive or

negative results often obtained on the "blank" tubes in an RIA procedure that requires extraction (14).

One way which is commonly used to check for cross reactivity in an assay is to run several levels of the possibly interfering substance in either a zero-dose or a low-dose patient and watch for a decrease in the B/F ratio. No change in B/F with increasing amounts of substance indicates that the compound does not compete with labeled antigen sufficiently to cause interference in the procedure.

Another procedure which may detect interfering reactions occurring in the assay is dilution of a high-dose level patient and assurance of parallelism to the standard curve. Although other conditions produce non-parallelism of patient dilutions, the presence of an interfering compound in the reaction mixture is a common cause of this discrepancy.

(d) Accuracy

The term accuracy has been defined by the IFCC as the "agreement between the best estimate of a quantity and its true value" (15). They state that it has no numerical value, but that its counterpart, inaccuracy, can be measured and expressed in the units of the quantity or as a percentage of the true value. One problem that arises in the clinical chemistry laboratory is the determination of what the "true value" actually is. For several reasons it is often impossible for the clinical analyst to arrive at a "true" value for an analyte. One reason is the fact that constituents analyzed

in the clinical setting are rarely in pure aqueous from, but are dissolved in solutions of varying pH, ionic strength, and protein content (i.e., serum, urine, or body fluids). These matrix differences may have differing and inconsistent affects on the test method, and may result in values which are not necessarily those which would be obtained if the analyte were in aqueous solution. Another common deterent to finding the "true" value of many protein analytes is the fact that the actual structure and molecular weight of the protein may not be known, and a pure protein standard may not be available. These problems are particularly common in RIA procedures even though steps have been taken to obtain relatively pure standards and dissolve them in as similar a matrix as possible to the sample specimens.

As an alternative to finding the "true" value, the clinical chemist must often rely on the value obtained by a reference method which has been shown to correlate well with clinical observations. This is the basis for the split-sample comparison analysis. In RIA the "reference" method can be a previous in-house procedure which has been clinically proven, or a procedure currently in use by another laboratory.

Accuracy of a procedure is really dependent on all three parameters previously mentioned--precision, specificity, and sensitivity. An insensitive assay will produce inaccurate values at low concentrations of analyte, while a non-specific antibody may yeild falsely elevated results. Imprecision or random error also affects accuracy since the larger the con-

fidence interval, the less chance of obtaining the actual value.

Accuracy (or inaccuracy) estimates analyze three types of error: random error; and the two components of systemic error--proportional and absolute errors. Procedures for the determination of random error are the same as those used to determine precision, i.e., replicate sample analysis. Proportional error can be estimated by recovery studies using a pure standard, absolute error by the addition of known interfering compounds, or total systemic error by use of split-sample analysis against a reference method (16). On a day-to-day basis accuracy (or more specifically, a change in accuracy) is monitored by assaying control samples with established values.

Although any factor which affects the precision, specificity, or sensitivity of the assay may also influence its accuracy, the most important and most commonly encountered condition in RIA procedures is the non-identity of standards and samples. This can result because the exact immunologically active structure of the biologically active molecule is not known, because different forms of the desired protein (such as precursor molecules or molecular fragments) are present in varying amounts in standard and sample, or because of matrix differences in standard versus sample. One of the commonly used methods to test for similar behavior of plasma hormone or protein and standard hormone is dilution of a high-dose patient and checking for parallelism to the standard curve. Accuracy assessment techniques such as parallelism

studies, split-sample analysis, and recovery studies will be dealt with in more detail below.

From the above discussion it can be concluded that the complete evaluation of a RIA kit is quite lengthy. In addition to the examination of the performance characteristics of the assay it is also useful to evaluate certain characteristics of the reagents themselves in order to find the sources of any problems that may arise. For example, we can look at the tracer to see if it is of good quality to try to discover the source of poor precision in an assay. Analysis of the antiserum may hint at problems in accuracy or sensitivity before these parameters are even evaluated. Other "tests" which may be performed on a new kit are: determination of tracer specific activity and immunoreactivity; plotting of a Scatchard plot to assess antisera properties; evaluation of non-specific binding; and observation of the shape of the dose response curve. A cost analysis and time study should also be included to conclude the overall practicality of the procedure.

It may be useful to make a preliminary assessment of the method using only some of the above criteria, and then decide whether it is necessary to proceed further (17). Since in RIA the antibody is the ultimate determinator of the sensitivity and specificity of the assay, it is often more valuable (especially when reagents are limited) to evaluate its' characteristics before obtaining voluminous data for precision

calculations. In this way, kits with inadequate qualities can be eliminated before unnecessary work is done. Exactly what makes a kit acceptable to use is discussed in the next chapter in more detail.

CHAPTER II

ACCEPTABLE QUALITIES IN KIT EVALUATION

Before discussing the "desirable" qualities of an RIA kit and its reagents, it must be mentioned that much information about the kit can be obtained from the package insert or from literature available from the manufacturer before actual performance of the assay. The IFCC has listed recommendations for the proper content of kit inserts or brochures (15). Of particular interest in RIA kit evaluation are facts about reagent composition, source, specificity, avidity, sensitivity, stability, and other specifications as appropriate. Comparison of antibodies of different kits can be made by looking at cross reactivity and accuracy studies performed by the companies; while the sensitivity of the assay and the value of K are also often available. Even though these parameters will be reevaluated in the laboratory, this information can be used to eliminate kits with grossly undesirable qualities. Information about the method of antigen labeling, purification techniques, or tracer specific activity may also be useful. A particularly high specific activity of a tracer, or a tracer that has been labeled by a vigorous method and has not been repurified to minimize damaged products, may predict problems arising because of low tracer immunoreactivity. Source, purity, and matrix of standards is also important information which may be used to predict problems present in assay accuracy. Reagent preparation and stability information may be significant

if test demand is low--both for assay quality and economic considerations. Specimen requirements including the effects of anticoagulants and preservatives on the results, and a list of precise experimental conditions which must be met such as pH, temperature, timing, or special manipulations, may indicate that the procedure is not practical enough to put into use. The necessity of reagents not supplied in the kit may also be a point to consider when choosing a procedure that meets the laboratory's needs. Literature references listed in the package insert are likewise valuable, since further research can easily be done if desired. Finally, not to be overlooked are normal range limits and the methods used to derive this data since normal ranges are not invariably the same with different antibodies.

Generally speaking, the more testing and research that has gone into kit development by the manufacturer, the more information will be available in the insert or from the company upon request. This fact may be a hint that the procedure may not be of sufficient quality or that it may need more intensive testing.

Below I will discuss some of the features which are desirable in an RIA procedure, particularly those concerning reagent characteristics. If reagents are of high quality, it follows that the performance characteristics will also be those of a quality procedure.

Antibody Characteristics

A "good" antiserum has the qualities of high titer, good specificity, and high affinity. Titer is a measure of the amount of antiserum used in the assay or the final dilution of antiserum used to bind the appropriate amount of labeled antigen in the absence of unlabeled antigen. It is the least important of the above mentioned characteristics. The advantage of an antiserum with high titer is that the antiserum can be diluted farther for use and will therefore last longer. This means that the lot number of antiserum from the manufacturer will change less often, and the necessity of reevaluation of the kit because of problems that may arise will become minimal.

The titer of antiserum produced in an animal against an antigen depends on several factors: 1) molecular size and composition of the antigen; 2) duration of the immunization; 3) dose of immunogen; 4) route of antigen administration; and 5) the "foreignness" of the antigen to the animal (18). There has been much controversy over the most efficient and productive method of producing high-titer specific antibodies with good affinity, but it seems that the best antisera are produced by high molecular weight antigens injected with Freund's adjuvant at multiple intradermal sites. The concentration of the immunogen should be minimal and the interval between injections maximal (19). Furthermore, a very pure antigen is not necessarily needed to produce adequate antisera, provided impurities are not structurally related to the immunogen.

Specificity is a property which primarily depends on the structure and/or binding characteristics of the antiserum itself. There are two common reasons for non-specificity of an antiserum: the presence of more than one species of antibody in the antiserum (heterogeneity); and an antibody which has been formed to a certain antigenic site of the antigen and which will react with that same functional group on a similar molecule (cross reactivity). Commonly, typical RIA antisera are not homogeneous, but do contain a population of different antibodies which may bind with the antigen and other undesirable molecules in the incubation mixture (see Fig. 3B). This heterogeneity presents no problem if the other molecules do not compete with the tracer for antibody sites; but, if they do, purification or extraction of the specimen to isolate the appropriate antigen may be required. Alternatively, specific absorption methods or affinity chromatography may be used to isolate the desired antibody.

The presence of similarly structured molecules which react with the same antibody binding site (Fig. 3A) is illustrated by RIA assays for the pituitary glycoproteins FSH and LH, TSH and HCG. Methods such as the inactivation of LH in FSH preparations using α-chymotrypsin (20), or the addition of HCG to the assay mixture to bind non-specific antibodies have been used to improve specificity. More specific antisera may also be obtained if antibodies can be induced against a more unique antigenic group, such as in the case of antisera developed against the β subunit of HCG (21).

FIGURE 3

NON-SPECIFICITY OF ANTISERA

Legend

A. There are several antigens (H) present which will displace tracer from the single antibody. Perhaps Ab_3 has been formed to a antigenic site on H_3 which is also present on H_2 and H_4.

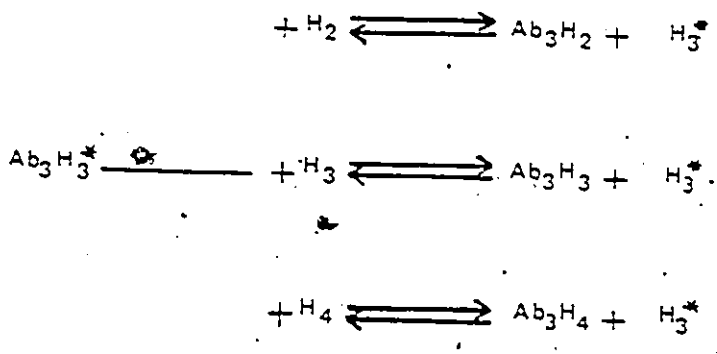
B. There are several different antibodies present in the antiserum which will react with labeled antigen. If their corresponding unlabeled antigens (H_4, H_6 and H_7) are present, they will cause cross reactions in the system and an undesirable assay.

Taken from: Odell, W.D., Abraham, G. A., et al.: "Production of Antisera for Radioimmunoassays", Principles of Competitive Protein Binding Assays (Odell W.D., Daughaday, W. H., eds.) J. B. Lippincott, Philadelphia. (1971), p. 70.

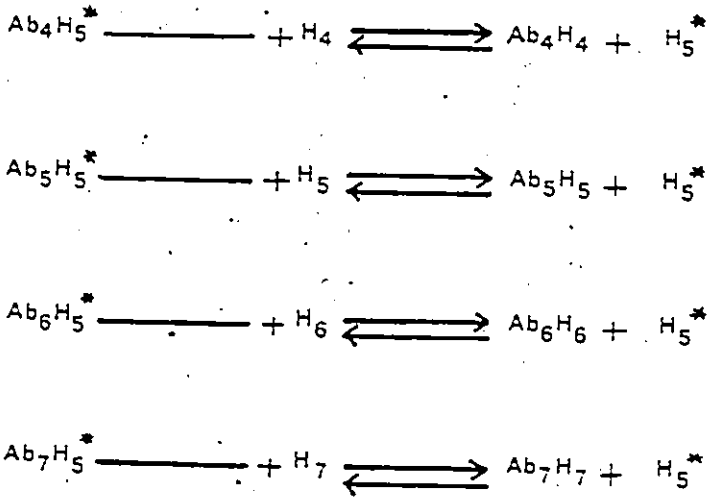
FIGURE 3

NON-SPECIFICITY OF ANTISERA

A.



B.



A relatively new breakthrough which may be of unlimited value in RIA is the technique of producing "hybridomas", or cells derived from a single lymphocyte which can be grown in tissue culture and allowed to produce large quantities of mono-specific antibodies (22). Another new technique is the use of endocrine tissue receptors as the binding agent (19). These receptors have the advantage of high specificity and avidity for the biologically active form of the hormone.

The affinity of the antiserum is defined as the energy of binding between the ligand and binder or the firmness of binding. It is commonly quantified as the value of K, and is the limiting factor to the sensitivity of the assay. Exactly why K limits sensitivity can be seen if we start with the definition of K given in Eq. 4, p. 3. It can be seen that if K is high then $[AgAb]$ must be much greater than $[Ag][Ab]$; or the bound antigen is greater than free and antibody sites are near to saturation. Saturation of antibody is necessary for unlabeled ligand to "compete" with labeled ligand and cause a significant (greater than 10%) change in the bound to free ratio of tracer (23). Stated differently, a small change in hormone concentration, ΔH, produces a large change in B/F, and the slope, $\Delta(B/F)/\Delta H$, is large at zero dose. If K is low, then there are more free antibody sites and more free tracer; thus, the addition of a small amount of unlabeled ligand causes a very small change in B/F that goes unnoticed. Resultingly, the slope of the curve is shallow. Since sensitivity can be thought of as precision near zero dose (24),

and precision depends on both the variance in the dose response variable and the slope of the dose response curve (10) (see also Fig. 2), an increase in slope at zero dose increases precision and therefore increases sensitivity.

Another fact to consider is that the above argument assumes that tracer concentration is adequate to saturate antibody, but is not too large to invalidate a small addition of unlabeled antigen. According to Ekins (12) this concentration is ideally A/K and B/F at zero dose of unlabeled antigen is unity (or 50% binding of tracer). The details of the effects of the tracer characteristics on assay sensitivity will be outlined below.

The concentration of the antiserum, or more specifically the number of binding sites available in the reaction mixture, also influences assay sensitivity since at lower concentration of antibody less antigen will be required to produce a detectable change in B/F. As a result, antiserum dilution is often used to increase the sensitivity of a procedure. Dilution of antiserum may also serve to increase assay specificity since lower affinity antibodies are diluted out and will have less influence on the assay (25). The disadvantage of antiserum dilution is that this technique tends to limit the range of the assay becuase antibody sites quickly become saturated with unlabeled antigen. It is often necessary, then, to compromise either sensitivity or range to obtain the assay which best suits the needs of the laboratory. Also, since the

speed of reaction is proportional to reagent concentration, dilution of antiserum may require an extended incubation period in order for the reaction to reach equilibrium conditions. A very good summary of the affects of K, antibody concentration, and tracer concentration on the shape of the dose response curve and the expected sensitivity and range is given by Feldman and Rodbard (13).

Normally, the stability of the antiserum is no problem especially if it is either kept frozen or in a lyophilized form (26). Unfortunately, storage conditions at the manufacturer may not be known so that antiserum may deteriorate. Also, the supplier may change the lot of antiserum without notification. These problems necessitate in-house checking of antiserum by Scatchard plots and parallellism studies at regular intervals.

Tracer Characteristics

Unlike the antigen used in the production of antisera, the antigen that is radiolabeled should be pure and of high quality (11). This is to avoid the formation of labeled molecules which do not compete with the desired unlabeled antigen, and may thus produce either non-specificity or poor sensitivity in the assay. Exceptions to the use of highly purified antigen for labeling can be made if 1) methods are available for the isolation of the radioligand after the labeling reaction, or 2) pure preparations of the contaminants are available and can be added in the assay in excess to prevent

labeled contaminants from binding to the antibody (27).

It should be mentioned that the antigen to be labeled may not need to be the whole antigen, nor of exact chemical structure as the desired antigen. The only requirement is that labeled antigen have homologous antigenicity with the unlabeled analyte, i.e., it reacts with the same antibody binding site (27). This also does not mean that the tracer have exactly the same affinity constant as the unlabeled antigen. Although theoretically K^* should be equal to K, practically it has been found that assay sensitivity is increased if K^* is slightly larger than K (12). On the other hand, if K^* is less than K (a phenomenon which may occur during the labeling procedure or as tracer ages) assay sensitivity is compromised.

The most commonly used radioisotopes in the clinical laboratory are ^{125}I and ^{3}H. ^{125}I has the advantages of a shorter half-life than ^{3}H and also a higher counting efficiency. It is often used in the labeling of protein molecules, since it readily reacts with the phenol rings of tyrosine residues. ^{125}I has replaced ^{131}I almost entirely because of its higher counting efficiency, longer half-life, availability in practically carrier-free form, lower gamma ray energy, and minimum expense. Procedures which are used to attach the radionuclide to the protein molecule include the chloramine T method, enzymatic labeling using lactoperoxidase, and conjugation labeling (27-29).

Tritium is most often used in the quantification of small

non-protein molecules which are present in relatively high concentration in serum (for example, steroid molecules). It does not alter the chemical structure of the antigen as greatly as ^{125}I because it is both small and can easily be substituted for a hydrogen atom (29). Its disadvantages are low specific activity and low counting efficiency. It is therefore not as commonly used as ^{125}I in the clinical setting.

Characteristics of the tracer which are commonly evaluated and are a judge of the quality of this reagent are specific activity and immunoreactivity. Specific activity is the number of radioactive atoms per molecule of ligand or the activity (usually in μCi) per mass of ligand. The advantage of a high specific activity tracer is to increase the sensitivity and precision of the assay. Exactly how sensitivity is increased by tracer specific activity is illustrated in figure 4. The high specific activity tracer requires less antibody to produce a given B/F ratio since less antibody sites are taken up by "non-labeled" tracer molecules. Therefore, antiserum can be diluted farther and assay sensitivity is increased (30). Also, the high specific activity tracer can itself be diluted farther and still produce statistically precise count rates. This diluted tracer again enables antiserum to be diluted farther and thus increase sensitivity. It must be remembered, though, that sensitivity is ultimately limited by K, and that increasing tracer specific activity does little to improve the sensitivity of an assay utilizing a binder with borderline low affinity (28). As will be seen

FIGURE 4

SPECIFIC ACTIVITY AND SENSITIVITY

Legend

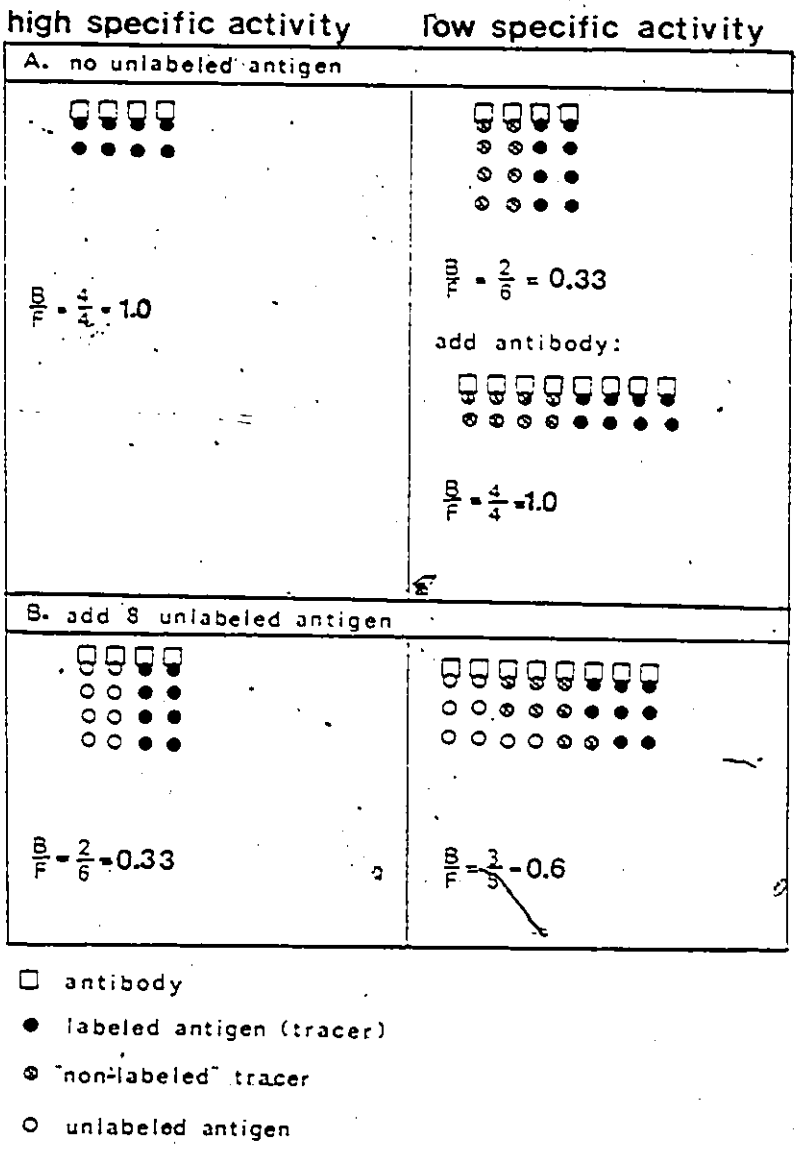
A. In the presence of no labeled antigen, a tracer of high specific activity (all ligand molecules are labeled) will produce a B/F ratio of 1.0 with the antibody that is present. A low specific activity tracer that contains several "non-labeled" tracer molecules only produces a B/F ratio of 0.33. In order to bring the B/F ratio to 1.0 more antibody must be added.

B. Addition of 8 molecules of unlabeled antigen to the system with the high specific activity tracer produces a B/F of 0.33. On the other hand, the B/F ratio produced in the system with the low specific activity tracer is only 0.6. The general effect of the poor tracer is to essentially "dilute" the unlabeled antigen so that it will not cause as great a displacement of labeled ligand.

Taken from: Goldsmith, S. J.: "Radioimmunoassay; Review of Basic Principles", Seminars in Nuclear Medicine, 5 (2), (1975), pp. 127,131.

FIGURE 4

SPECIFIC ACTIVITY AND SENSITIVITY



increasing specific activity may in fact compromise the assay even further by producing damaged tracer molecules.

The maximum obtainable specific activity is limited by the fact that more than one radioactive atom per ligand molecule increases the probability that the labeled ligand will become chemically or immunologically altered. This alteration may be due both to the incorporation of extra or different atoms into the molecule which make the tracer structurally different from unlabeled antigen, and to radiation damage that may occur to the labeled ligand when the radionuclide releases energy as it decays ("decay catastrophe") (31). Generally it has been found that an average of 0.5 to 1.0 ^{125}I atoms per molecule of ligand has little affect on K^*, yet provides sufficient tracer specific activity (27). There are exceptions to the rule, though, and optimal specific activity for individual antigens should be determined experimentally.

The immunoreactivity of the tracer is also a very important characteristic of this reagent since it is necessary that the labeled ligand react similarly to unlabeled ligand in a "competitive" assay. Normally, if a tracer has been iodinated properly and has been purified to remove any damaged molecules or free iodine, excess antibody should bind 90 to 98% of the radioactivity present (26). Non-bindable counts can be due to either free iodine or damaged ($K^* \ll K$) tracer molecules. Damaged molecules can be produced either from the iodination reaction, from spontaneous damage that occurs to the protein

molecule as the tracer ages, or from degradation of tracer from enzymes and other components of plasma during the incubation period of the assay. The development of more sensitive assays has minimized tracer damage during incubation since the small amount of serum or plasma that is necessary for the assay is diluted enough in the final reaction mixture to have little affect on the tracer. Assays which use a final plasma dilution of 1/10 or greater usually have no problem with incubation damage (26).

The useful life of the tracer depends on several things including: half-life of the radioisotope, nature of the ligand that is labeled, and initial specific activity. Average life is about one to two months but varies and should be determined for each individual labeled analyte. Tracer damage (especially from aging) is usually first seen as a decrease in B/F of the zero tube, a decrease in assay sensitivity, a flattening of the dose response curve, and an increase in assay imprecision (26). The lowered B/F is due to the decrease in tracer immunoreactivity either from "unlabeled" tracer molecules or from damaged labeled ligand molecules which have lowered antibody affinity ($K^* \ll K$). These same phenomena produce the lowered assay sensitivity (see Fig. 4) and flattened dose response curve. Damaged tracer may also have abnormal adsorption to separating agents or test tube walls (25). This adsorption may be variable and contributes, along with the flattened dose response curve, to greater imprecision and decreased reliability of results.

Purification of tracer after iodination, or if necessary to rejuvenate an old tracer, is usually done by gel filtration on Sephadex columns (28). This procedure separates labeled protein from free ^{125}I and excess iodinating reagents, and if a long enough column is used, will separate damaged protein components (29). Other methods which have been used are dialysis, adsorption chromatography, ion-exchange chromatography, and gel electrophoresis (28).

The amount of tracer that is used in the assay is also crucial, particularly to the sensitivity of the procedure. Theoretically, tracer should be adequate to just saturate a high affinity antiserum, or should be approximately the same molar concentration as the antibody (25). In reality, though, a lower affinity antiserum is often used and it may be necessary to add an excess amount of tracer to achieve adequate saturation. It has been found empirically and with computer models that sensitivity is actually maximum when the amount of radioligand markedly exceeds the antibody binding capacity (25). Best sensitivity is obtained at about 50% binding where there is optimum saturation yet minimum error. Adding a larger amount of tracer also serves to improve counting statistics since increasing total activity will increase both bound and free counts. The limitation of this approach is that "misclassification error" (32), or errors produced in the separation step, are also increased. These errors are especially seen as greater activity in the non-specific binding (NSB) tubes. In assays with high bound fractions the effect of a large NSB

may not be too important; but as B/F decreases, this error may have quite a significant effect and may produce large errors of precision and accuracy in the high dose range of the curve.

Separation Techniques

In RIA the molar concentrations of the reactants are usually too low to favor the spontaneous precipitation of multiple antigen-antibody complexes. Therefore, techniques have been developed to separate the bound and free fractions to enable individual quantification of each. The ideal separation technique is able to partition bound and free radioactivity rapidly and completely without disturbing the antigen-antibody binding reaction, and can accomplish this without being affected by constituents in the incubation mixture which are unrelated to the analyte. Failure to separate fractions completely and accurately has been designated as "misclassification error", and can have a large influence on the precision and accuracy of the assay (3).

The most commonly used separation techniques in the clinical laboratory are the "tube" types and include: double-antibody precipitation; adsorption methods; chemical precipitation; and solid-phase separation (33). Less commonly used techniques are based on the differential migration of bound and free fractions and include chromatoelectrophoresis and gel filtration. In chromatoelectrophoresis undamaged free hormone adsorbs at

the site of application, while bound hormone, damaged hormone, and free iodide move under the influence of an electric current and buffer flow caused by evaporation (11,33). The advantage of this process is that damaged labeled antigen and free iodide can be separated to allow for correction of individual samples. However, the complexity, expense, time required, and high tracer specific activity necessary prohibit its routine use.

Gel filtration uses the molecular volume difference between bound and unbound antigen to selectively allow small molecules to pass into gel particles while inhibiting passage of large ones. Sephadex and Biogel are commonly used gels (33). The final incubation mixture is either passed through a column under conditions that allow elution of the bound moiety or the gel is allowed to equilibrate and is present during the whole procedure. Equilibration methods are often used in competitive protein binding assays since they do not disturb the binding of antigen and antibody and are relatively independent of time and temperature. Problems with gel-filtration methods are the time and space required and the difficulty in the collection of several eluted fractions--a procedure which is necessary for optimal separation of fractions.

Tube-separation methods can be grouped roughly into those that precipitate or bind the antibody bound antigen, and those that adsorb or remove free antigen. The techniques are based upon either differences in molecular structure and size, or immunoreactivity between the bound and free antigen as a

consequence of the large molecular weight antibody coupled to it. Methods which bind or adsorb the free antigen are plagued by the fact that they often disturb the binding equilibrium (especially with low affinity antisera) by removing free antigen and pushing the reaction in a direction that replaces the unbound component. Timing is, therefore, very critical in these procedures and should be as equal as possible from tube to tube. Use of low temperatures (4° C) also partially corrects for the "stripping" effect by maximizing the affinity constant of the binding reaction. The adsorption methods using such materials as charcoal, talc, kaolin, and powdered silica (QUSQ) are typical free-antigen removal techniques.

Another critical factor in using these adsorbers is the concentration of protein in the reaction mixture. Excess protein may cause a decreased adsorption of the free antigen, while low protein may produce an increased adsorption of the antigen-antibody complex. Protein content of individual tubes should, therefore, be standardized either by addition of excess protein to all tubes, or by use of small sample sizes to minimize any variations in protein content in different samples. Adsorption of the bound complex when using charcoal has also been minimized by coating the charcoal particles with dextran of appropriate molecular size. The pores provide openings for penetration of unbound antigen, but inhibit passage of antibody-bound complexes. The necessity of dextran coating of charcoal has been under question, though, and several investigators

believe that when protein concentration is adequate (10 to 30%), the dextran has no advantage (34). Other variables which may affect the amount of binding by the adsorber are pH, temperature, surface area of the adsorber, and ionic strength. These factors should be optimized for each procedure. The main advantage of using these techniques to separate bound and free fractions are low cost, speed, and simplicity.

Double-antibody precipitation employs a second antibody to precipitate the soluble antigen-antibody complexes. The second antibody is usually an anti-gamma globulin produced against the animal species supplying the primary antibody, and can thus be used in any assay in which the first antibody was formed in that species. Similar to the first antiserum, the precipitating antiserum must also be used at its optimal dilution. Also, extra "carrier" non-immune globulin such as rabbit or sheep serum is added to enhance the formation of precipitable aggregates (32). Under optimal conditions, this separation technique fulfills most of the desirable characteristics mentioned earlier. It is very specific, gives complete separation of bound and free antigen, does not usually disturb the binding equilibrium, and can be used in virtually all RIA procedures. Since it requires a second immunological reaction, problems do arise because of differences in protein concentration, the presence of complement, and other non-specific effects due to the presence of serum. To avoid some of these problems, non-immune globulin or hormone-free serum is added to all tubes to equalize protein concentration, and

EDTA or heat inactivation is used to eliminate complement interferences (35). Increasing incubation time for the precipitation step also serves to minimize the effects of interfering factors.

Double-antibody techniques can also be used as "pre-precipitated" methods. In this system the first and second antibody are reacted before addition to the incubation mixture. It has the advantage that the second antibody reaction is completed separately in a controlled environment so that non-specific effects are minimized. Excess carrier globulin does not have to be added and incubation time is shortened. One disadvantage, though, is that the sensitivity in this method is often less than that of the "post-precipitation" methods, probably as a result of steric blockage or chemical alteration of the primary antibody binding site due to its attachment to the precipitating antibody (35). The main disadvantages of all double-antibody precipitation techniques are their high cost, and the requirement of an additional incubation period.

Chemical precipitation of the antigen-antibody complex can be accomplished by "salting-out" with ammonium sulfate or sodium sulfate, or by lowering the solubility of the complex with such organic solvents as ethanol, dioxane, or polyethylene glycol. Immunoglobulins have the lowest solubility of all proteins so that the antibody is precipitated while most small polypeptides, including free antigen, stay in solution. Problems with this type of separation method are that the amount of free antigen that is precipitated along with immunoglobulin

depends upon the nature of the antigen, the concentration of the precipitating agent, and the amount of protein present. As a result, conditions must be optimized for each procedure to assure satisfactory and complete isolation of bound and fractions. Advantages are the ease and rapidity of the technique, and the fact that no additional incubation period is required.

Solid phase procedures are those in which the first antibody is covalently bound or adsorbed to an insoluble polymer (such as dextran), beaded agarose (such as Sepharose), the inside of plastic or polypropylene tubes, or polypropylene disks. Separation is achieved rapidly and simply by centrifugation and/or decantation and washing. Other advantages are long storability, good reproducibility, and economy. Also, the fact that binding of antigen and antibody is virtually irreversible, and is not disturbed by the separation procedure, allows for shorter incubation times. The problems with this technique are that antibody avidity may be changed because of steric blockage of the binding site or chemical alteration of the antibody molecule. Additionally, because one of the reagents is not moving through solution, or is only moving slowly, a lower association rate constant or speed of reaction results. This may decrease sensitivity (36). Significant loss of antibody titer has also been reported, thus requiring larger amounts of antisera. Sources of error may arise due to variability of batches of coated polystyrene tubes, or from extensive washing of the binder which may dissociate

antigen-antibody complexes (33).

One point which deserves discussion is the behavior of damaged radioligand during the separation procedure. Radioligand which has been altered during the iodination reaction, because of prolonged storage, or during assay incubation, will usually bind to antibody with less affinity and will remain in the unbound fraction. It may behave differently, though, during separation, and may non-specifically coprecipitate with antibody in methods that precipitate the bound fraction. The same is true of free iodide which may be present in the damaged tracer solution. Free iodide and altered radioligand may also unpredictably bind to adsorbers which precipitate free ligand, and will therefore bias the B/F estimation (32). Evaluation of the behavior of damaged tracer is best accomplished by studying the immunoreactivity of the trace and the NSB tube of the assay.

Standard Characteristics

It is essential that in RIA the substance to be measured and the standards be immunologically identical or indistinguishable in their behavior in the assay system (11). Ideally, the standard is a "pure" sample of the desired analyte, is well characterized as far as molecular structure and weight, is stable during isolation, transport, and storage in the laboratory, and is available in large enough quantities to be used for a long time in several different laboratories (37).

The problem that arises in radioassay determinations is that the exact molecular structure and weight of many peptide molecules is not known, and the biologically active form or forms of the substance cannot be adequately isolated from biological materials. Attempts to produce highly purified peptides may be complicated by variations in batches of starting materials, or by changes that may occur during isolation and purification. For example, preparations of glycoprotein hormones may differ in their content of carbohydrate and extent of sialation. Also, purified hormones sometimes tend to form dimers and polymers upon purification. If the peptide structure is known and the hormone can be synthesized, there is a large possibility, particularly in molecules composed of 20 or more residues, of errors in sequencing or conformational structure. These errors could affect the antibody binding site and, thus, immunoreactive identity of the molecule. A further complication is the existence of several biologically similar immunologically different forms of the desired analyte (38). Variations in the proportions of these different forms in sample versus standards is another source of inaccuracy in the assay.

The purified standard should be stable during shipment to the laboratory or during storage. Freeze-dried preparations may vary in moisture content or in stability. Repeated freezing and thawing may disrupt protein conformation. Total protein content of the standard preparation should be adequate to prevent adsorption of the analyte to the walls of the container,

but should not contain proteolytic enzymes which may degrade the standard substance.

There are several types of standards or reference preparations which can be used in the assay procedure. International standards are collected, tested, and aliquoted by the World Health Organization International Laboratory for Biological Standards. They are the most reliable of preparations, and have been extensively tested for potency and stability, but are usually not available in sufficient quantity for routine use. Their potencies are given in either units of weight if the molecular weight of the hormone is known, or in terms of biological activity. Reference materials have not been tested as extensively as international standards, but have certain values of purity and stability provided by the producer. Such institutions as the Division of Biological Standards, National Institute of Medical Research, London, and the National Institute of Arthritis, Metabolic And Digestive Diseases (NIAMDD), Bethesda, Maryland, distribute such preparations. "Working" reference preparations are those which are most commonly used in routine assays, and are usually calibrated by use of an international standard under the same assay conditions and with the same antiserum as that used in the routine assay protocol. In kit procedures, this calibration is done by the manufacturer.

Not only should the standard hormone be immunologically identical with the sample hormone, but it is important that the matrix of both sample and standard be as similar as possible

to eliminate non-specific factors which may affect the antigen-antibody binding reaction or the separation procedure. To insure this, standards are often diluted with hormone-free serum or albumin preparations. The affects of protein and other components on the separation procedure have been discussed previously, whereas the influence of pH, ionic strength, and other variables on the primary antigen-antibody binding reaction will be discussed under the section on reaction conditions.

One of the most commonly used methods to check for similar behavior of standard and unknown is dilution of a patient sample and demonstration of parallelism to the standard curve. It should be emphasized, though, that this is not absolute verification of identity, but is a requirement (11). Ekins discusses the observation that molecules of different chemical structure or physical configuration may react with the same antibody binding site and produce curves similar enough so as not to be distinguished from each other (12). This fact may give rise to serious accuracy errors of the assay.

Reaction Conditions

Besides the antigen, tracer, antibody, and separation reagent, other variables in the reaction mixture may influence both the primary antigen-antibody reaction and the separation system. Antigen-antibody reactions generally dissociate at

extremes of acidity or alkalinity, but are usually independent of pH from 7.4 to 8.6 (11). Consequently, buffers that are most often used in RIA procedures are: 0.04 or 0.05 M phosphate, pH 7.4; 0.04 or 0.05 M Tris, pH 7.4; 0.01 or 0.02 M Veronal, pH 8.6; and 0.1 M borate, pH 8.4. Often 0.1 or 0.2% BSA or HSA is also added to the buffer to prevent antigen tendency to bind to glassware, tubes, pipettes, etc. Low ionic strength buffers are preferable since high salt concentrations have been shown to interfere in immune reactions. Ionic and pH variation can be introduced from differences in sample composition; but this can be minimized if sensitivity is adequate to determine unknown concentrations in a 1 to 10 or greater plasma dilution.

Substances such as anticoagulants, bacteriostatic agents, and enzyme inhibitors may also interfere with the antigen-antibody reaction. Heparin has been shown to decrease assay sensitivity in some procedures because of its existence as a strong polyanion (39). The bacteriostatic reagent merthiolate and Trasylol, an enzyme inhibitor, may also produce immune reaction interference (31). If these agents are present in both standards and unknowns, the assay may still be valid but may have reduced sensitivity.

Protein concentration variations mainly affect the separation system of the assay, particularly when the method is based on the adsorption of free antigen. Protein decreases binding of the free antigen to the adsorbant, but inhibits the non-specific adsorption of the bound complex. Protein concen-

tration may also affect the double-antibody precipitation method by changing the bulk of the precipitate formed. If sensitivity is adequate to allow sufficient dilution of samples, and/or protein is added in optimal concentration to all tubes, these effects are minimized.

It has been found that K is dependent on changes in temperature: $\Delta F^{\circ} = -RT \ln K$, where ΔF° is the standard free energy change, R is the gas constant, and T is absolute temperature (40). Thus, the temperature at which the assay is carried out has an effect on the immune reaction. The equilibrium constant is generally greater at 4°C than at room temperature. This is due to the fact that the dissociation rate is often more temperature dependent than the association rate. Slowing dissociation by decreasing temperature then increases the association/dissociation ratio (K) (25). It has also been shown experimentally that the energy of the antigen-antibody binding reaction increases at low temperatures (25). Conversely, equilibrium is reached faster at higher temperatures (room temperature or 37°C), so that assays sometimes employ initial incubation at room temperature or 37°C followed by incubation at 4°C to decrease assay time yet maximize K. Higher temperatures may increase damage to labeled and unlabeled ligands during incubation, though, thus decreasing their binding capacities.

Although the initial rate of the antigen-antibody reaction is rapid, equilibrium is approached relatively slowly (25). Extending incubation time slowly increases total binding, but

also shifts the binding from one of relatively rapid dissociation to one of slow dissociation rate. The change may depend on a transfer from a rapidly equilibrated and dissociated order of binding sites to a second order with low dissociation. Another possibility is that aggregate formation between several antigen-antibody complexes may alter the kinetics of the binding reaction. The final result is seen as an increase in the avidity of binding and a concomitant increase in assay sensitivity. This is why sensitivity increases as incubation time increases.

At this time, many assays are performed as "non-equilibrium" procedures for the simple reason that extended incubation times are often not practical. Since equilibrium is not reached and the incubation time is shorter, the exactness of timing, especially between the first and last assay tubes, is often critical. The only step where this causes major problems is in the centrifugation step, since all tubes do not have reagents added at the same time but must be centrifuged simultaneously. This problem may produce a limit to the number of assay tubes that can be run in one assay set-up.

An alternative to the competitive system in which both cold antigen and tracer are allowed to react with antibody at the same time is the sequential or non-competitive assay system. This method requires preincubation of unlabeled antigen with antibody, and has the advantages of increasing assay sensitivity and decreasing the amount of time in which the tracer is in contact with serum. Decreased contact time

is desirable if tracer is particularly sensitive to degradation by serum constituents. Sequential assays are also very useful in conditions where labeled and unlabeled ligands have differing antibody affinities (41). As in competitive assays, increasing incubation time further increases sensitivity, but care should be taken to prevent reestablishment of equilibrium between tracer and unlabeled ligand. This in effect negates the sequential-addition conditions. Ekins indicates that incubation times can be optimized similar to optimization of reagent concentrations for any desired target ligand concentration, but usually requires utilization of computer techniques (3). One drawback of sequential addition methods is a decreased assay reproducibility (especially when reactant concentrations are low) produced by minor differences in handling conditions and timing which cause major differences in the amount of labeled antigen-antibody complex formed (41).

A point which is often overlooked when considering both incubation times and the use of sequential techniques is the specificity of the assay that results. Pratt and Woldring have indicated that reduced specificity is found under conditions where reaction equilibrium is not reached (i.e., when using short incubation times) and under conditions where antibody is not saturated (in sequential addition techniques). These conditions permit more cross reaction by interfering antigens by making more antibody sites available to them (42).

As mentioned earlier, timing is also important in the separation step if a free antigen adsorbant is used. Both

total time that the reaction mixture is in contact with the adsorbant, and the difference in time between the first and last tube of the assay should be controlled as closely as possible to prevent variable and non-specific adsorption by the separating agent.

CHAPTER III

METHODS OF EVALUATION

Least Detectable Dose

The least detectable dose (LDD) is a practical assessment of the obtainable sensitivity of the assay. As mentioned in previous sections, sensitivity may be regarded as precision at zero dose, and precision is dependent on both the slope of the dose response curve and the uncertainty in the estimation of the dose response variable. Taking these points into consideration, if we calculate the 95% confidence interval at zero dose, we can estimate what the lowest concentration is that can be detected which is significantly different from zero. Therefore, the zero tube (no unlabeled ligand) is assayed several times within a run, and the mean and standard deviation of the dose response variable (Y) is calculated. LDD is the dose read from the curve at Y which is minus 2s from the mean of the zero tube (see Fig. 5A).

Rodbard suggests that a better approach is to use a one-sided students t-distribution (43) and check for a significant difference between the zero dose and a low-dose patient. He also suggests inclusion of a factor which accounts for uncertainty in the position of the dose response curve. The formula he uses is:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{(s_1^2/n_1 + s_2^2/n_2)}}$$

FIGURE 5

DETERMINATION OF LEAST DETECTABLE DOSE

Legend

Three methods are commonly used to determine LDD of an assay.

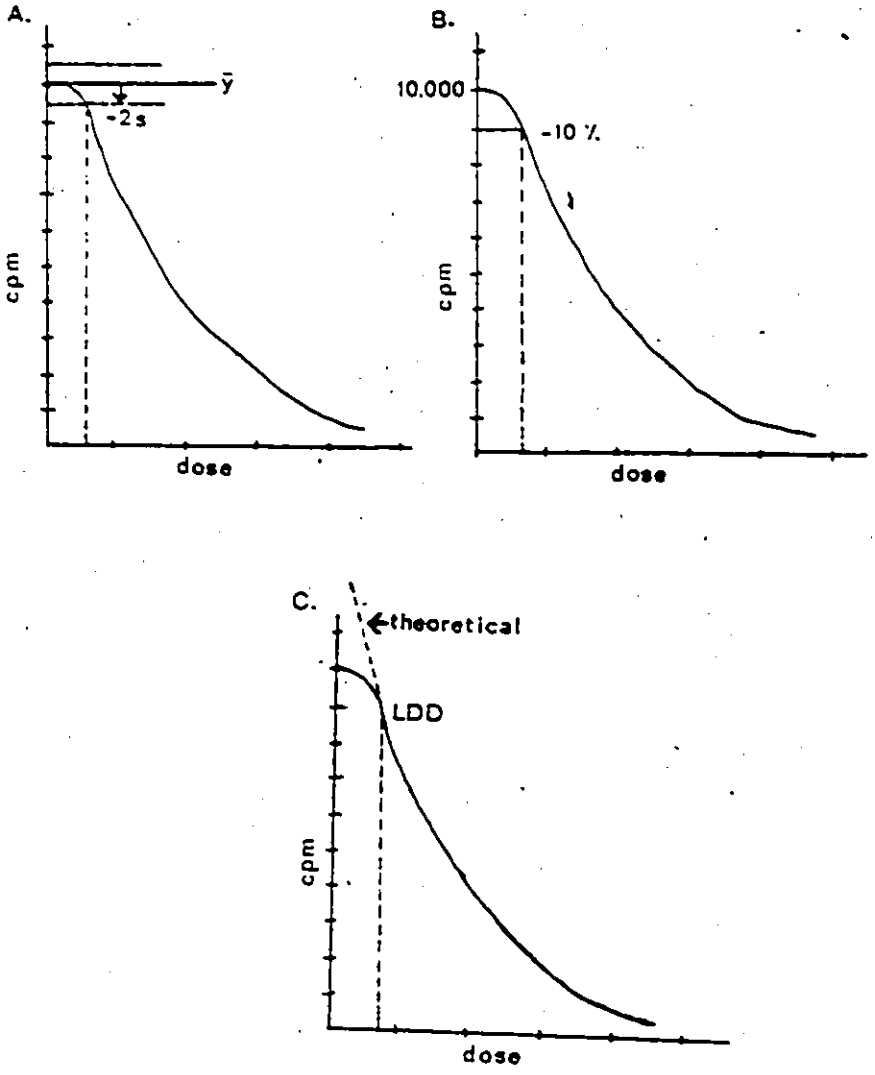
A. Replicate analysis of the zero-dose tube will provide data to calculate mean and standard deviation of the dose response variable. Mean CPM minus 2s will give LDD interpolated from the curve.

B. Alternatively, mean CPM minus 10% of this mean can be used.

C. Dilution of the lowest standard will also give an idea of LDD, but it is important to consider precision of both the zero tube and the assayed dilution. Ideally, the dose response curve of CPM vs. dose is a hyperbola. Deviations from this form near zero dose will produce non-linear dilutions of the low standard.

FIGURE 5

DETERMINATION OF LEAST DETECTABLE DOSE



where $\overline{Y}_1$, s_1, n_1, and $\overline{Y}_2$, s_2, n_2 are the mean response, standard deviation, and number of replicates for the zero dose and for the minimal detectable dose, respectively. $\overline{Y}_2$ is statistically different from $\overline{Y}_1$ at the desired probability level when the t-ratio calculated is greater than the tabulated percentiles of a students t-distribution. LDD is then determined by dose interpolation from the standard curve.

It was also mentioned that a "significant" change in the B/F ratio was often taken to mean a greater than 10% difference (26). This is another way of estimating LDD, or if replicate analysis of the B_0 tube is not possible, it is a quick check on the assay sensitivity. This time the a value obtained is dependent only on the slope of the dose response curve and the mean value of Y at B_0. It is found as the dose comparable to: $\overline{Y}_{B0} - 0.10\,\overline{Y}_{B0}$ (see Fig. 5B).

An actual determination of LDD may be made by dilution of the lowest standard available in the kit. LDD is the highest dilution at which the curve begins to lose linearity (see Fig. 5C). The precision of the curve at this point must also be taken into consideration to see if the resolution is sufficient to differentiate this dose from zero. As a general rule, whichever method (precision of the zero tube or dilution of the lowest standard) yields the higher LDD value should be taken as the sensitivity of the assay.

One phenomenon which may be discovered when checking sensitivity by dilution of the lowest standard is known as the "low-dose hook". Antibodies normally have two antigen

combining sites per molecule, which in RIA are assumed to be independent of one another. This is not necessarily true, though, and several studies have shown that binding of one antigen may alter the binding properties of the second combining site to make binding of the second antigen more favorable (44,45). This "cooperativity" is seen as a maximum in the dose response curve at low concentrations of ligand (see Fig. 6). The occurrence of these B/F values which are greater than B/F of the zero tube accounts for the negative values sometimes obtained at low concentrations of analyte.

Non-specific Binding

Non-specific binding (or the "blank" count) is considered to be any radioactivity present in the bound fraction which is not due to reaction with the primary antibody (35). The reasons for NSB are varied and also depend upon the type of separation procedure utilized. For example, at the low concentration of reagents used in radioligand assays, there is a tendency for proteins, including tracer, to adsorb to any type of solid material. This adsorption could be to the glass walls of a test tube, in which case the NSB would be increased in procedures which precipitate the bound fraction. In procedures which precipitate free antigen, this adsorption may go unnoticed, since it is the supernatant bound ligand which is counted. The use of plastic test tubes or increased amounts of albumin or protein to prevent non-specific adsorption

FIGURE 6

POSITIVE COOPERATIVITY

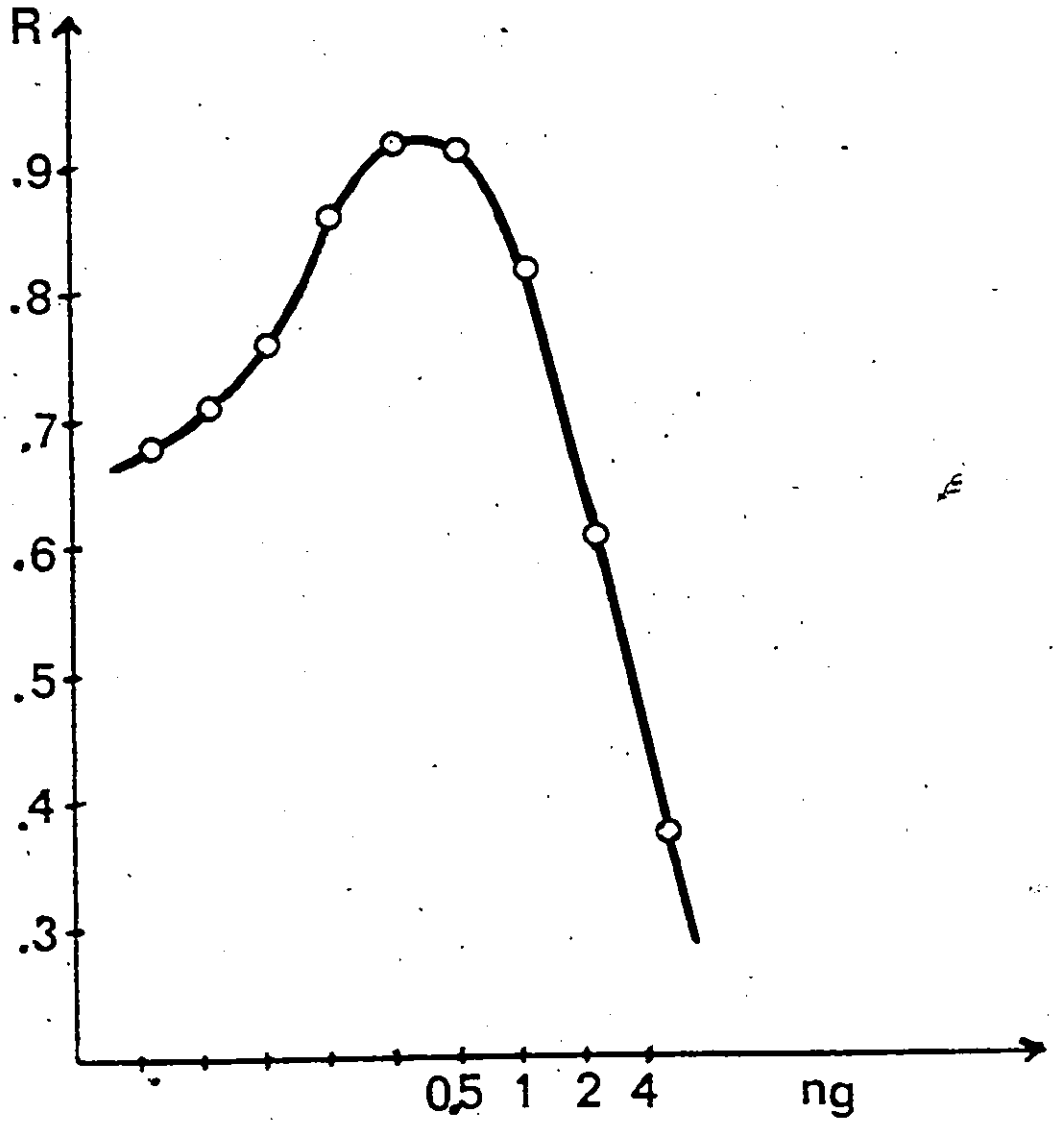
Legend

R is the ratio of antibody bound antigen to free antigen. This data shows the typical "low-dose hook" or positive cooperativity phenomenon believed to be due to a modification in binding affinity of one antibody site caused by binding of antigen to the other site of a bivalent antibody molecule.

Reproduced without permission from: Neiderer, W.: "The Interpretation of Paradoxical Radioimmunoassay Standard Curves", Journal of Immunology Methods, 5, (1974), p. 79.

FIGURE 6

POSITIVE COOPERATIVITY



may decrease NSB.

Non-specific binding is determined by either eliminating the binding agent of the assay or by adding excess cold ligand to saturate it. The second procedure is more desirable (though seldom used) since the presence of antibody changes (usually reduces) non-specific binding. Normally, the NSB tube is set up with the zero standard or a protein matrix, tracer, and precipitating agent. The counts obtained are subtracted from all subsequent tubes. There is a problem with this method, though, since "patient" NSB may vary because of differences in cold ligand concentrations, differences in matrices of samples, variances in tracer degradation by different sera, or presence of endogenous antibodies. As a result, NSB should be checked with many different serum samples and with all standards to determine consistency. Only then can it be assumed that the subtraction of NSB obtained from only one tube is valid.

In separation procedures which produce a very heavy precipitate, free antigen may become trapped and cause large values of NSB (25). This may necessitate washing of precipitate. Other "misclassification" errors of radioactivity may result from insufficient centrifugation of solid adsorbents which leaves particles in the bound supernatant, or decanting errors which leave excess free antigen droplets with the bound precipitate.

Large NSB values may also be produced because of excessively damaged tracer which may non-specifically precipitate in

the bound fraction, or may have decreased binding to solid adsorbents. Damage which is produced during incubation may vary between samples and standards, and thus should be checked with several different sera. It should be noted, though, that antibody binding of tracer usually protects it from damage so that performance of NSB without antibody may overestimate tracer damage (25).

The presence of endogenous antibody will increase patient NSB, except in double-antibody separation procedures. In these cases, the free fraction contains the tracer bound to endogenous protein and final results will appear larger than actual. Also, non-specific binders present in normal serum may bind tracer and cause high NSB values, particularly when tracer becomes damaged. Included are binders such as albumin, and α_2-macroglobulin (46). These binding proteins also interefere with the antigen-antibody reaction, may alter the binding kinetics, and may give rise to serious accuracy errors. If this problem of unknown binders becomes too serious, the binding proteins may have to be denatured by proteolytic enzymes or heating. Alternatively, the binding capacity of the protein may be saturated with an analog of the analyte, or the analyte may have to be extracted from serum.

It is clear, then, that the patient NSB's must be checked to verify similarity to the standard or zero-dose NSB. If the two are different, steps must be taken to either correct individual results or eliminate the cause of the discrepancy. If they are similar, minimizing NSB in general is necessary.

As mentioned above, high NSB values may not have a large effect if the B/F ratio is high; but in the high-dose range of the curve where B/F is low, serious errors may result as a consequence of a large NSB of tracer.

Tracer Immunoreactivity and Specific Activity

Immunoreactivity of tracer can be assessed in one of two ways. The first method comprises adding a large excess of antibody to a mixture of zero standard and tracer, and determining the maximum percent of the total radioactivity that is bound. Ideally, 90 to 100% binding is obtained with the non-bindable radioactivity assumed to be non-immunoreactive tracer (26). Usually non-immunoreactive labeled ligand is due to damaged tracer formed because of iodination, incubation damage, or aging; but may also be because of radioactive impurities in the tracer reagent. Non-binding may also be produced if the binding reaction does not reach equilibrium, or if the bound antigen is "stripped" from the antibody by the separation procedure.

The exact immunoreactive mass can also be determined by using increasing amounts of tracer as an unknown sample. The "self-displacement" or drop in B/F which results can be used to read mass of trace from the standard curve (Fig. 7). The procedure for performing and calculating tracer self-displacement is given in APPENDIX A. Failure of any significant B/F drop with increasing amounts of tracer means either

FIGURE 7

TRACER SELF-DISPLACEMENT

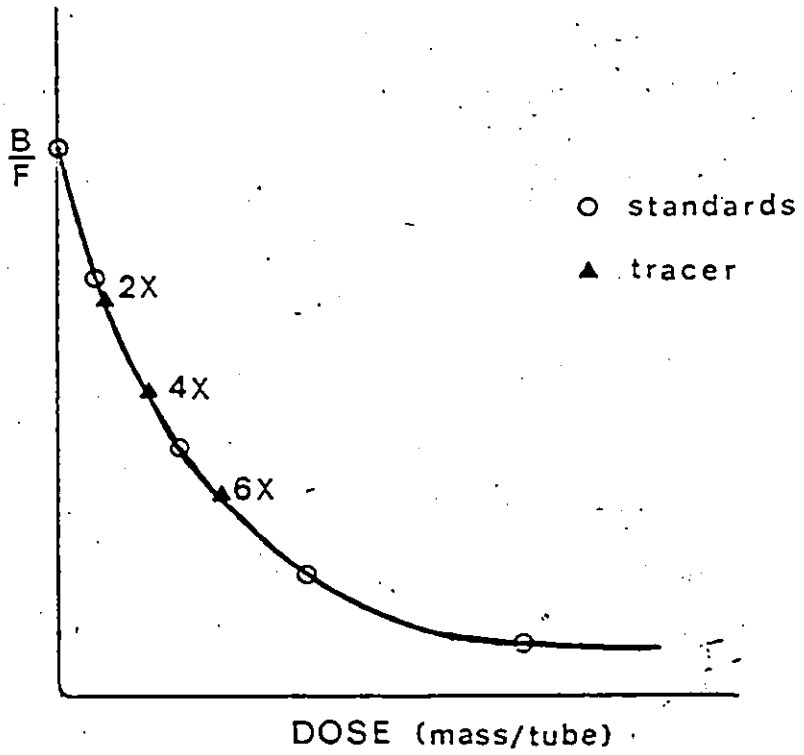
Legend

Addition of increasing aliquots of tracer will cause a decrease in B/F which should be parallel to that of the standard curve. The average mass of immunoreactive tracer can be calculated from values interpolated from the standard curve.

Reference: Kagan, A.: "Evaluation of Commercial Radioimmunoassay Kits", Seminars in Nuclear Medicine, 5 (2), (1975), p. 177.

FIGURE 7

TRACER SELF-DISPLACEMENT



a significant alteration in tracer immunoreactivity (tracer damage) (26) or a very low tracer mass. This procedure not only gives an idea about the immunoreactive mass of the tracer, but also indicates whether tracer reacts similarly to standards i.e., $K_{std} = K^*$. Plotting the data in Scatchard form will allow assessment of parallelism by comparison of the slopes of the lines.

Once immunoreactive tracer mass has been calculated, the "immunoreactive" specific activity may be determined by using the formula:

$$\bar{\sigma} = \frac{T}{r \cdot E \cdot L_s}$$

where $\bar{\sigma}$ is specific activity, T is total counts per minute per tube, r is the conversion constant between DPM and μCi (2.2×10^6 DPM/μCi), E is counter efficiency in CPM/DPM, and L_s is immunoreactive tracer mass in mass/tube (47). Comparison of calculated specific activity with that obtained from the manufacturer may give an idea of tracer damage that has resulted in the interim.

Specific activity values obtained from the manufacturer are either determined by the above procedure or by analysis of the reaction mixture immediately after iodination (29). Since known amounts of radioiodine are used in the labeling procedure, separation of unreacted iodine from labeled antigen will indicate what percent of the total radioactivity was incorporated into the mass of antigen. Techniques such as paper or thin-layer chromatography and electrophoresis are used for separation. The major problem with this method is

that it assumes that all antigen is labeled and that there are no degradation products formed or that the products are separated completely from labeled ligand. Calculation of specific activity by analysis of the reaction mixture can then lead to erroneous values if degradation or unlabeled products are present. This may account for discrepancies in specific activity calculated in the lab by tracer displacement versus that obtained from the kit manufacturer.

The Scatchard Plot

In 1949 Scatchard described a method for determining the attraction or affinity constant of small molecule/protein interactions (48). This calculation was later applied to the antibody-ligand reactions of radioimmunoassay by Berson and Yalow (49). The logic behind Scatchard plots and their use begins with the assumption that the reaction between antigen and antibody is governed by the law of mass action. We take the basic reaction of antigen and antibody (Eq. 1; p. 2):

$$Ag + Ab \underset{k_r}{\overset{k_f}{\rightleftharpoons}} AgAb \qquad \text{(Eq. 1)}$$

The equilibrium constant can then be given as (Eq. 4, p. 3):

$$K = \frac{k_f}{k_r} = \frac{[AgAb]}{[Ag][Ab]} \qquad \text{(Eq. 4)}$$

If $[Ab^0]$ is the original concentration of antibody, and $[Ab]$ is the concentration at equilibrium, then $[Ab] = [Ab^0] - [AgAb]$. $[AgAb]$ is the bound antigen and can be indicated as B, while

free antigen, $[Ag]$, is given as F. Substitution into equation 4 gives:

$$K = \frac{B}{F([Ab^0] - B)} \qquad \text{(Eq. 6)}$$

and,

$$B/F = K([Ab^0] - B) \qquad \text{(Eq. 7)}$$

This is recognizable as a linear equation: $y = mx + b$, so that if B/F is plotted on the y-axis and B on the x-axis, the slope of the line will be -K, the y-intercept $K[Ab^0]$, and the x-intercept $[Ab^0]$ (50) (see Fig. 8A). This type of transformation of data is valuable because both the equilibrium constant, K, and the antibody concentration, $[Ab^0]$, can be estimated (13).

In RIA there are two methods used to obtain a Scatchard plot. The first utilizes increasing amounts of tracer and no cold ligand, and plots B*/F* versus bound mass of tracer. This may be easy to do for manufacturers who have an unlimited supply of tracer available to them, but may be very impractical for the routine clinical laboratory which is trying to evaluate a kit. An alternative method is use of increasing amounts of cold ligand in place of tracer, and estimation of total bound ligand by looking only at bound tracer. The complete calculation of bound mass, B, and B/F by use of the standard curve (assay conditional Scatchard plot) is given in APPENDIX B (47). With this method we are also making the assumptions that B*/F* represents the distribution of both labeled and unlabeled ligand, $K^* = K_{std}$, and the fraction B^*/T also reflects the

FIGURE 8

SCATCHARD PLOTS

Legend

A. The theoretical Scatchard derived from use of the mass action law is a straight line. Useful values obtainable from this method of plotting data are: -K (the slope), and $[Ab^0]$ (x-intercept).

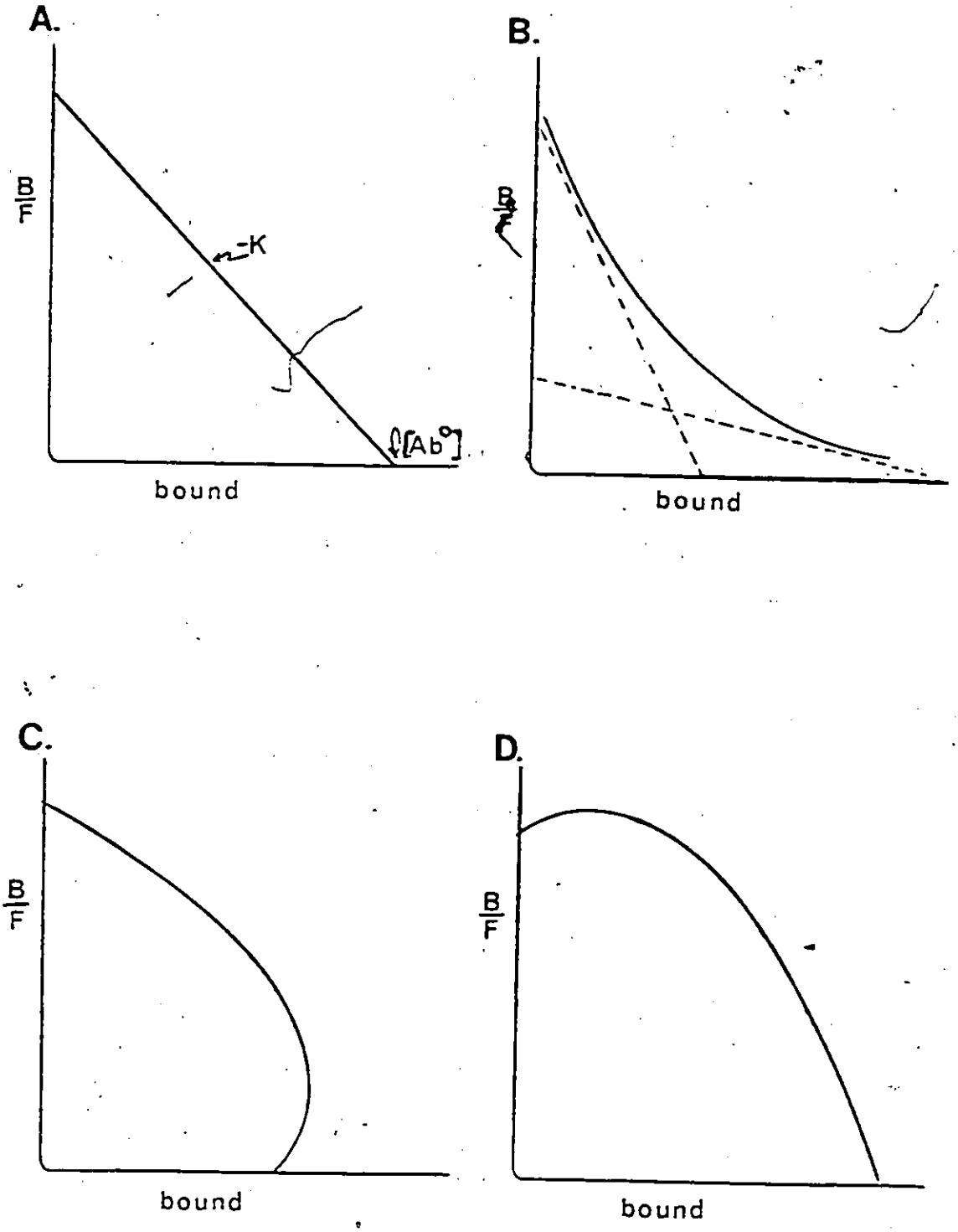
B. Concave plot results from a heterogeneous antibody mixture. The curve can be resolved into two components (dashed lines) with different binding affinities and different concentrations.

C. Artifactual Scatchard backbending is produced because of damaged tracer or non-equilibrium conditions.

D. This Scatchard shows the positive cooperativity phenomenon.

FIGURE 8

SCATCHARD PLOTS



fraction of total mass bound:

Theoretically, if all assumptions about binding are made as mentioned above (p.10) (13), a straight line should result. Unfortunately, because of several factors including the heterogeneity of antisera and of ligands (unknown cold antigen vs. labeled antigen), a straight line rarely results in Scatchard plots of RIA data. The first case that often results is shown in figure 8B. This concave type of curve has been most often explained as the existence of more than one species of binding site. The curve can be resolved into two or more straight lines, produced by sties of different concentrations and different equilibrium constants, which add to produce the non-linear plot (50-52). When the standard curve is used to generate the Scatchard plot, an explanation for this right skewing has been given as the presence of labeled ligand which binds more avidly than the unlabeled species ($K^* \gg K_{std}$) (53). In this case, the mass bound estimation (x-axis) based on the formula: B/T (mass tracer + mass standard) is over-estimated because of a larger than expected B^*.

It has been suggested that if a long "tail" is produced in this type of Scatchard plot, the binder causing this is of low affinity and high capacity, and may be considered as non-specific binding (54) which should be subtracted from all bound values obtained. Chamness and McGuire also indicate that the amount of non-specifically bound ligand is proportional to the amount of free ligand present in the mixture. As

indicated in this study, though, this correction may cause problems if applied to every case where a right-skewed Scatchard plot is obtained.

The second type of non-linear curve which may result is shown in figure 8C. In this case there is a left skewing or a backbending. The exact cause and significance of this has not been decided upon, but at least two theories have been proposed. Hollemans and Bertina (55) have concluded that backbending of the curve results from a labeled ligand with binding affinity less than the unlabeled ligand ($K^* << K$)--a condition which often results from damage to tracer. Another possible explanation of this type of plot is that equilibrium conditions have not been reached, the bound fraction then being lower than expected and bound mass being underestimated.

Another less commonly encountered Scatchard plot results from the same conditions that produce the "low-dose hook" seen in plots of B/F vs. dose. Positive cooperativity at very low ligand concentrations will produce a comparable maximum in the Scatchard plot (Fig. 8D) (56).

A point to mention is that if a heterogeneous antibody and damaged tracer are both present in a system, a linear Scatchard plot may be obtained. For this reason, Scatchard plots should not be interpreted too strictly if other parameters of the kit evaluation yield conflicting information.

A further check on antibody characteristics as observed from the Scatchard plot is to increase and decrease antibody

concentration and verify parallel plots (57). Non-parallelism may indicate a shift in the predominant binding antibody, or give information about optimal reagent concentrations. Parallel Scatchard plots serve to corroborate the equilibrium constant calculated using the normal amount of antibody.

Parallelism Studies

As indicated earlier, one of the primary conditions necessary for validation of an RIA procedure is that standard and unknown react similarly with antibody, or verification that the apparent concentration of an unknown is independent of the dilution at which it is assayed (11). Non-superimposability of the standard curve and dilutions of a high-dose patient can arise from several causes including: chemical interference with the antigen-antibody binding reaction (matrix effects); degradation of labeled antigen because of serum constituents; use of heterologous standards; and presence of cross-reacting substances (11). Parallel studies will not indicate the cause of the discrepancy, but only that a problem exists.

Typically, a high-dose patient is diluted in a protein matrix or hormone-free serum, and is run simultaneously with a normal standard curve. The curve resulting from patient dilutions is plotted on the same graph as that of the standard curve. Several graphical methods are available (see Fig. 9), but only plots of B/B_0 vs. log dose and logit Y vs. log dose permit easy determination of parallelism, and only the logit

FIGURE 9

PARALLELISM ASSESSMENT

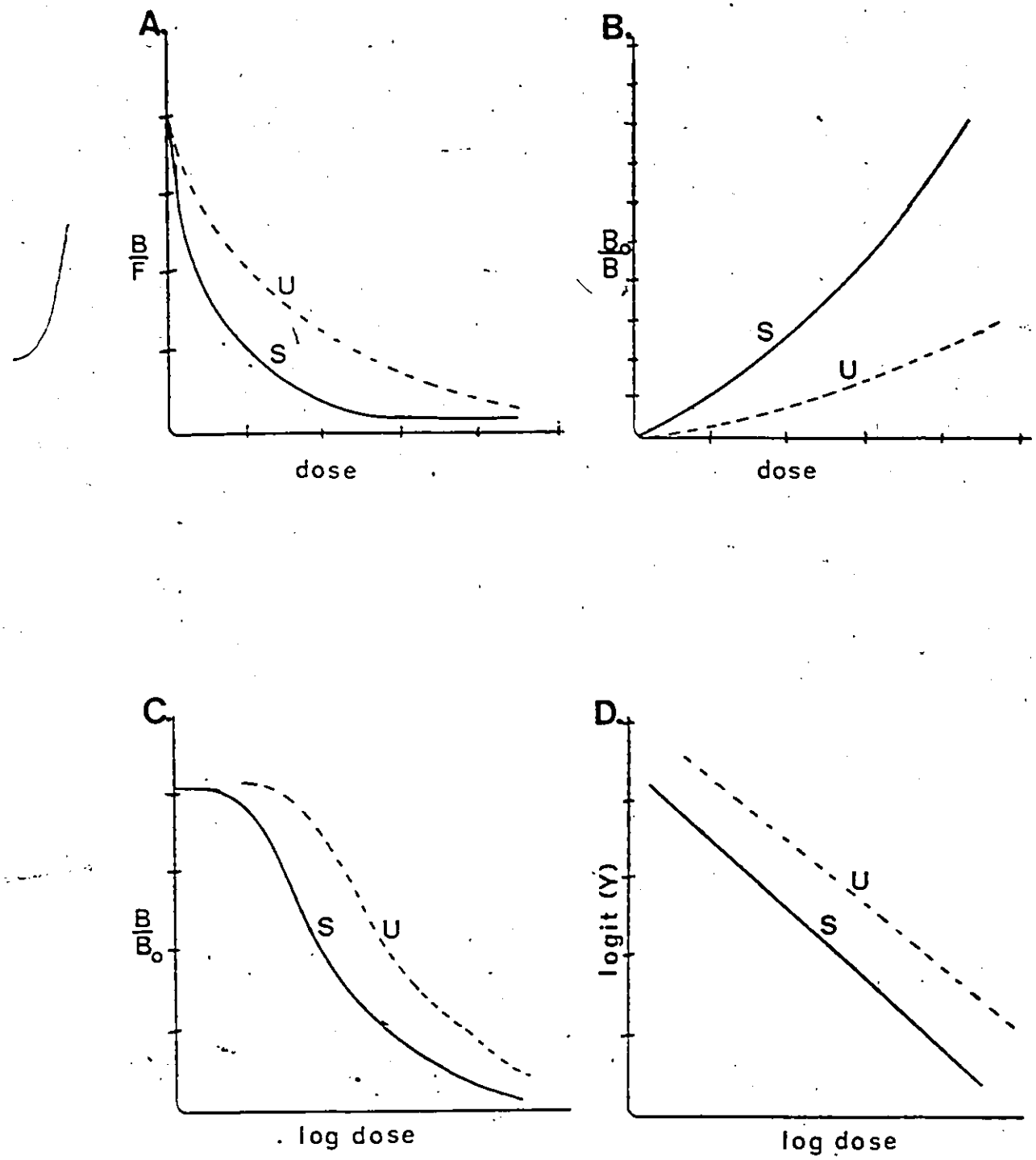
Legend

These graphs indicate several graphical methods available for analyzing radioimmunoassay data. Theoretical inhibition curves for standards (S) and unknown (U) are presented for comparison. Graphs C and D allow easy comparison of parallelisms, and only D (logit transformation) produces straight lines amenable to statistical analysis.

Reproduced without permission from: Midgley, A. R.: "Principles for The Assessment of The Reliability of Radioimmunoassay Methods (Precision, Accuracy, Sensitivity, Specificity)", Karolinska Symposia on Research Methods in Reproductive Endocrinology, (1969), p. 165.

FIGURE 9

PARALLELISM ASSESSMENT



transformation provides straight lines for slope comparisons. It is important to note that the logit transformation, although it allows for linearity, does so at the expense of increased non-uniformity of variance--particularly at the high and low ends of the curve (10). Thus, the method of "weighting" points of the curve by use of the reciprocal of the variance must be used so that significant statistical conclusions can be reached (10).

Another method that may be utilized to analyze patient dilution results is plotting of the expected dose vs. the actual dose. Linear regression will yield a slope of one if the curves are parallel (43). Again, allowances should be made for the imprecision of the determination. Finally, a plot of the result of the dilution times dilution vs. dilution should yield a line parallel to the x-axis (58). It is important in this instance to also plot the confidence intervals of the obtained values to compensate for precision errors. One major flaw with this type of graphical analysis is that precision errors at low doses (and high dilutions) tend to be magnified out of proportion.

It must be reemphasized at this time that parallelism does not necessarily unequivocally confirm identity of standard and unknown. As Ekins has indicated, dissimilar species may produce curves which are so close in parallelism that they appear identical (12). This may cause results which are in error by several factors of magnitude.

Recovery Studies

Recovery studies have been a generally accepted method for assessing accuracy of RIA procedures (59). As mentioned earlier, though, these studies are valuable only in assessing proportional error or bias (17) and not constant bias. For this reason, recovery determinations should not be the only assessment of procedural accuracy, but should be supplemented by split-sample comparisons against a reference method. It is assumed in recovery studies that 1) the pure substance used as the "spike" is actually pure and of known chemical structure, 2) the amount of pure substance added can be weighed out accurately, and 3) the concentration of ligand in the base can be accurately measured. If these assumptions are valid, then factors which affect recovery may be: choice of protein matrix used in the study; imprecision of the assay; sensitivity of the assay; separation procedure used; accuracy of the standards used to produce the standard curve; and method used to calculate recovery results.

Matrix choice may influence the primary antigen-antibody reaction or the efficiency of the separation procedure due to differences in standards and recovery samples. Assay imprecision should be taken into account when plotting standards and when determining sample results to minimize any bias that may arise. Sensitivity may affect results when they approach very low dose levels, especially if non-specific binding in the base is high. The choice of separation proce-

dure may result in misclassification errors which could cause errors in B/F estimation. A large source of poor recovery in RIA procedures is inaccuracy of standard values, especially when the exact nature and structure of the desired analyte in not known.

There are two common methods used to calculate recovery results. Method #1 calculates total analyte found (C) divided by total analyte expected, where the expected amount is equal to the amount added (A) plus the amount already present in the base (B):

$$R_1 = \frac{C}{A + B} \times 100$$

This method usually gives higher recovery values, and is not as sensitive to errors of endogenous analyte in the base or to errors due to non-specific binding (59). Method #2 calculates recovery as analyte recovered (measured value, C) minus base value (B), divided by the amount of spike added (A):

$$R_2 = \frac{C - B}{A} \times 100$$

This method is more sensitive to proportional errors at the high and low ends of the standard curve.

Recovery data can also be plotted as total amount found (C) vs. total amount expected (A + B), and linear regression analysis applied. This may yield information pertaining to the type of error that may be present in the assay. The ideal slope is 1.0 with y-intercept of zero and correlation coefficient, 1.0. Deviation of the slope from unity indicates a proportional error perhaps due to matrix effects. A non-zero

intercept could raise suspicion of inaccuracy in the base measurement, errors due to standard inaccuracy, or non-specific binding in the base. A low correlation coefficient suggests random errors either from poor assay precision or poor sensitivity (poor precision at low concentrations) (60).

The typical preparation of spiked samples for recovery analysis begins by weighing a pure analyte and dissolving it in an appropriate solvent to form the "stock" solution. An aliquot of stock solution is added to a protein base and serial dilutions are made. This method eliminates errors in pipetting different (and small) amounts of stock solution into base, but may produce an error if the initial addition is inaccurate.

Bases which can be used include: a zero standard obtained from another kit or pooled from several kits; a patient serum with low ligand concentration; BSA or HSA solutions; a charcoal-stripped serum pool; a heterologous serum; or "simulated" serum containing albumin and TGB (for the globulin component). Problems arising when using a low-dose patient as base may be due to a large degree of variation between individuals in terms of effect on antigen-antibody binding reaction. Human or bovine serum albumin solutions may have large influences due to differences in ionic strength or pH in serum vs. albumin solutions. Charcoal-stripped serum pools (61) may contain small amounts of suspended charcoal which may interfere with recovery. Stripping may also alter protein constituents of the pool since non-specific adsorption by the charcoal removes

variable amounts of protein.

Recovery samples, a sample of base, and standards are analyzed simultaneously, and recovery data are calculated from values obtained by interpolation from the standard curve. Several recovery assays should be performed using separate recovery sample dilutions to eliminate pipetting errors. Also, if no method is available to verify the exact concentration of the stock solution (such as spectrophotometrically), several preparations of the stock solution should be used.

As indicated, the exact molecular weight and structure may not be known for some analytes (especially protein hormones), or the substance may not be available in pure form. In this case, an alternative method for recovery studies is to use an unknown patient plasma and add aliquots of standards from the kit. The expected result would be:

$$\frac{(\text{standard value} \times \text{volume added}) + (\text{unknown value} \times \text{volume})}{\text{total volume}}$$

The unknown value is determined by use of the standard curve. Recovery can then be calculated as: (observed result/expected result) x 100. Data may also be plotted as observed vs. expected, and regression analysis applied (62).

Cross Reactions

Any substance which binds to the same site on the antibody as the ligand being assayed will cause displacement of tracer and false analyte values. Cross reactions in RIA procedures

are usually evaluated by assaying increasing amounts of the suspected interfering compound and looking for a drop in B/F. It is important in this instance to use a similar matrix to that normally found in standards and unknowns. It is also important to use concentrations of cross-reacting substance that could possibly be found in sera to be analyzed. For example, growth hormone (hGH) reacts with rabbit antisera formed against HPL (63). However, the importance of the cross reaction is minimized because the sensitivity of the antiserum to HPL is 1000 to 10,000 times greater than to hGH (64), and the concentration of HPL in pregnancy sera is nearly 1000 times the concentration of hGH (μg/mL *vs.* ng/mL).

Cross reaction, or the potency of the interfering compound, may be estimated in at least four different ways.

(A): The 50% displacement method expresses cross reaction as the ratio of the amounts of antigen needed to cause a 50% drop in the initial B/F ratio, *i.e.*, $(B/F)_0/2$, where Y is the desired analyte and Z is the cross-reacting substance:

$$\text{cross reaction of Z} = \frac{Y_{50}}{Z_{50}} \times 100$$

This method is the most often used, but it has the disadvantage that it underestimates errors. This is because it takes little account of the region where highest cross reaction occurs, *i.e.*, at low unlabeled antigen concentrations. It has been found that cross-reacting antigens usually displace tracer from the least specific antibodies of a heterogeneous antisera before reacting with the specific antibodies. Thus, at low

antigen concentrations, the desired analyte is competing with tracer for specific antibody sites, while at the same time, cross-reacting substances are displacing tracer easily from non-specific sites. This yields a steep initial cross reaction curve which becomes nearly horizontal at higher antigen concentrations (65).

(B). The CR_{1ng} method expresses cross reaction as the ratio of displacements caused by one nanogram of both antigens:

$$\text{cross reaction of Z} = \frac{(B/F)_Z}{(B/F)_Y} \times 100$$

This method may give more reliable cross reaction estimates; however, 1 ng is an arbitrary amount and does not often correspond to assay conditions (65). Also, if equivalent amounts of both antigens are used, they may not reflect physiological concentrations which may be present in unknown samples.

(C). The 10% error method expresses cross reaction as the ratio of the initial gradients of the two displacement curves:

$$\text{cross reaction of Z} = \frac{Y_{50} \times 0.10}{Z_{(10\% \text{ error})}} \times 100$$

$Z_{(10\% \text{ error})}$ is that amount of Z which causes a 10% error in the assay of Y. It is determined by finding $Y_{50} \times 0.10$ (10% of the amount of Y needed to produce a 50% drop in B/F) and drawing a line from this point parallel to the dose axis and intersecting the Z curve. The corresponding dose on the Z curve is $Z_{(10\% \text{ error})}$.

This method gives realistic estimates of cross reaction at the

most relevant point--at the low dose range where cross reaction is maximal (65).

(D). Relative Reactivity (RR) is calculated as the mean concentration of desired analyte which is equivalent in reactivity to one concentration unit (for example 1 μg/mL) of the cross-reacting compound. A value of 1.99 for RR would indicate that 1.99 μg/mL of analyte produces the same response as 1 μg/mL of cross-reacting compound. An alternative is to calculate 1/RR, the reciprocal of RR, as the number of concentration units of cross-reacting compound which has the same reactivity as one concentration unit of analyte (66).

Graphical analysis and simple visual evaluation is also commonly used after dose response curves are plotted on the same graph for both analyte and cross-reacting substances. This method is used particularly to illustrate the absence of cross reaction by various compounds.

Split-Sample Analysis

Often the only method available to check on the accuracy of an assay, especially in RIA procedures where pure standard materials are sometimes not available, is by split-sample analysis using either a reference method or a clinically validated procedure. Comparison of results of the two methods may, in fact, be a better judge of accuracy than recovery

studies, particularly if the already established method has been confirmed to be a reliable and clinically accurate assay.

Samples are run as unknown in both the "reference" method and the "test" method. Data obtained is analyzed by several statistical methods: linear regression analysis, t-test, and correlation coefficient calculation. Reference values can be plotted on the x-axis with test results on the y (60). Linear regression analysis and subsequent calculation of slope (m), y-intercept (b), and the standard error of the estimate in the y direction (S_y), will give clues to proportional, absolute, or random errors respectively (60,67). Use of the t-test and calculation of bias and standard deviation of the differences, SD_d, will indicate constant and/or proportional error for the first, and random and/or proportional error for the second parameter (60,68). Calculation of the correlation coefficient, r, yields information about random errors (60,68,69). APPENDIX C contains formulae of the common statistical tests used in split-sample comparison studies.

Observation of the Dose Response Curve

Beginning with the definition of the equilibrium constant, K, and using equations derived in terms of p, p^*, and q, where p is the concentration of unlabeled ligand, p^* is the concentration of labeled ligand, and q is the concentration of binding sites, we can form a general quadratic equation which expresses the relationship between R, the dose response

variable B/F, and the concentration of cold ligand (12,13):

$$R^2 + R(1 + Kp_s + Kp^* - Kq) - Kq = 0$$

Assuming one antigen and one antibody, this equation can be rearranged to predict the shape of any dose response curve. More complex models have also been derived for more than one species of binding site and more than one ligand (13).

Several coordinate systems have been used to plot dose response curves including the more common: B/F, B/T, T/B, B/B_0, and bound counts versus dose or log dose. Plots of B/F, B/T, B/B_0, and counts bound versus dose give hyperbolic curves; while plotting these dose response variables versus log dose yield sigmoidal curves. Examination of these types of plots may indicate areas of "sensitivity" of the assay, or areas where precision may be poor.

With the advent of computers in the clinical laboratory, it has been desirable to attempt to linearize these hyperbolic and sigmoidal curves so that dose interpolation may be done automatically, thus increasing efficiency and decreasing turn-around time. Partial linearization of the hyperbolic plots may be obtained by use of the reciprocal of the response variable (for example T/B) (70), although this method magnifies error in the response variable for high doses. Linearization of sigmoidal curves may be achieved by either truncation of the low and high ends of the curve, or more commonly, by use of linearization models such as the logit-log transformation (71). The logit transformation is defined as:

$$\text{logit}(Y) = \ln\left(\frac{Y}{1-Y}\right)$$

where Y is either B/B_0 or $(B/F)/(B/F)_0$. The RIA dose response curve can then be described by the linear equation (72):

$$\text{logit}(Y) = Y' = a + b \log Y$$

where b is the slope of the line and a is the y-intercept. Logit-log plotting usually yields excellent linearity; however, when there is severe antibody heterogeneity, this model may fail to provide adequate linearization (13). Alternatively, if the computer contains non-linear regression capabilities, a parabola may be fit to the data using the method of orthogonal polynomials (73). As mentioned, use of the logit-log method magnifies the typical non-uniformity of variance seen in RIA dose response curves (74). Use of weighting functions established over a number of assays allows proper "fitting" of the curve (10).

Other methods which have been used for analysis of RIA data using computer "fitting" of curves include: weighted and unweighted logistic models (75), polygonal interpolation (73), and spline functions with and without smoothing factors (76). These methods require more sophisticated computer systems; and are, therefore, not as commonly used as the logit-log method. These methods may provide better accuracy, though, particularly at points removed from the standard values (73).

When evaluating a new RIA kit, it is desirable to plot the data in several ways to determine possible problem areas of the curve, or to decide upon the coordinate system which provides the most information and is best for the assay (73).

Practical Observations

Other factors which determine the acceptability of an assay are: ease of the procedure; shelf life of reagents; equipment necessary; turn-around time; assay time; and cost. A procedure which requires elaborate diluting equipment or special handling procedures may be impractical for the small laboratory. Assays with long turn-around times may be useless if results are needed on a "stat" basis. Reagents which require special storage or have short shelf lives could produce economic unfeasability, particularly if test demand is low. All of these parameters must also be evaluated objectively and weighed against the reliability of the assay results. It may be necessary to choose a kit with, perhaps, less precision and sensitivity for one with faster turn-around time or greater simplicity if results obtained yield clinically significant information.

2 CHAPTER IV

HUMAN PLACENTAL LACTOGEN

Human placental lactogen (HPL) or human chorionic sommatotropin is a peptide hormone that is very similar in structure to growth hormone, and consists of an almost spherically coiled protein filament of approximately 21,500 molecular weight. Both hormones contain 191 residues with two intramolecular disulfide bonds in exactly the same positions, i.e., between positions 53 and 165, and between 182 and 189. Examination of the amino acid sequences reveals that 161 of the 191 are identical in the two hormones. Of the remaining 30, 19 are highly compatible substitutions, four are relatively compatible, and only seven amino acid differences cannot be easily explained (77).

Prolactin is another protein hormone that is similar in structure to HPL and hGH. It contains 198 amino acids, two intramolecular disulfide bridges in relatively the same positions as HPL and hGH, a third disulfide bridge in the amino end of the molecule, and 146 residues which appear to correspond closely to portions of the hGH and HPL molecules. The structural similarities of these three hormones provides a strong indication that they evolved from a single progenitor molecule. It is not surprising, then, that HPL has been found to have effects in the body common to both hGH and prolactin (78).

Early studies with HPL extracted from human placentas resulted in similar sommatotropic actions with hGH, although

HPL had much weaker action. Experiments with rats showed that HPL caused an increase in the width of epiphyseal cartilage after the animals had been hypophysectomized. Further studies, though, with purer placental extracts, indicated that the function of HPL is probably one of potentiation of hGH activity. This could result from either slowing of growth hormone degradation, or saturation of certain binding sites by HPL, thus allowing more hGH to be available for other binding sites. But, whether HPL itself actually has growth hormone activity, or whether it only enhances hGH effects, the metabolic results of HPL produced during pregnancy are very similar to the actions of growth hormone.

HPL, like hGH, has anti-insulin properties (79). This antagonism, which is probably at the peripheral tissue level, causes a compensatory increase in insulin production by the pancreatic beta cells. This fact explains the higher than normal insulin levels found in pregnant women, and also accounts for the observation that pregnancy often converts pre-diabetics to overt diabetics. In these cases, the stress on the beta cells is enough to exhaust its capabilities of insulin production. HPL also acts to mobilize fats by increasing lipolysis, and produces a positive maternal nitrogen balance. The exact reasons for these metabolic alterations are not certain but could be 1) to make more glucose available to the fetus for its energy source or for the stress of labor and delivery, 2) to enhance protein synthesis for fetal development, and 3) to shunt the maternal energy source from glucose

to fat--a more abundant and calorigenic energy source (78,79).

HPL also possesses lactogenic and mammotropic properties similar to prolactin. HPL, in combination with insulin and hydrocortisone, increases synthesis of casein, α-lactalbumin, and β-lactoglobin by increasing RNA synthesis in the mammary gland cell. It also causes increased amounts of lactose and the appearance of milk in the mammary ducts, and causes increased weight of the mammary glands (78).

HPL also has luteotropic properties, but it has been suggested that it only acts as a cofactor with HCG in the promotion of gestational function (78).

HPL is produced during pregnancy by the cytoplasm of the syncytiotrophoblastic villous epithelium within the placenta. The placenta seems to produce one to three grams per day in the second half of pregnancy and three to twelve grams per day at term (80). Levels in the maternal circulation are detectable at about five weeks (about 25 ng/mL), rise rapidly during the first 30 to 34 weeks of pregnancy, and then fall during the final weeks (see Fig. 10). Normal levels in the maternal serum are about 1.5 µg/mL at 20 weeks and 6 µg/mL at 38 weeks (78). During the last four weeks before delivery, values show more variability than earlier in pregnancy. It should be mentioned that in calculating normal HPL ranges, the distribution of values is not Gaussian, but is slightly skewed to the high end. This fact makes the discovery of low HPL levels in a pregnant woman even more significant (81). It should also be mentioned that very little

FIGURE 10

NORMAL VALUES OF HPL IN MATERNAL SERUM

Legend

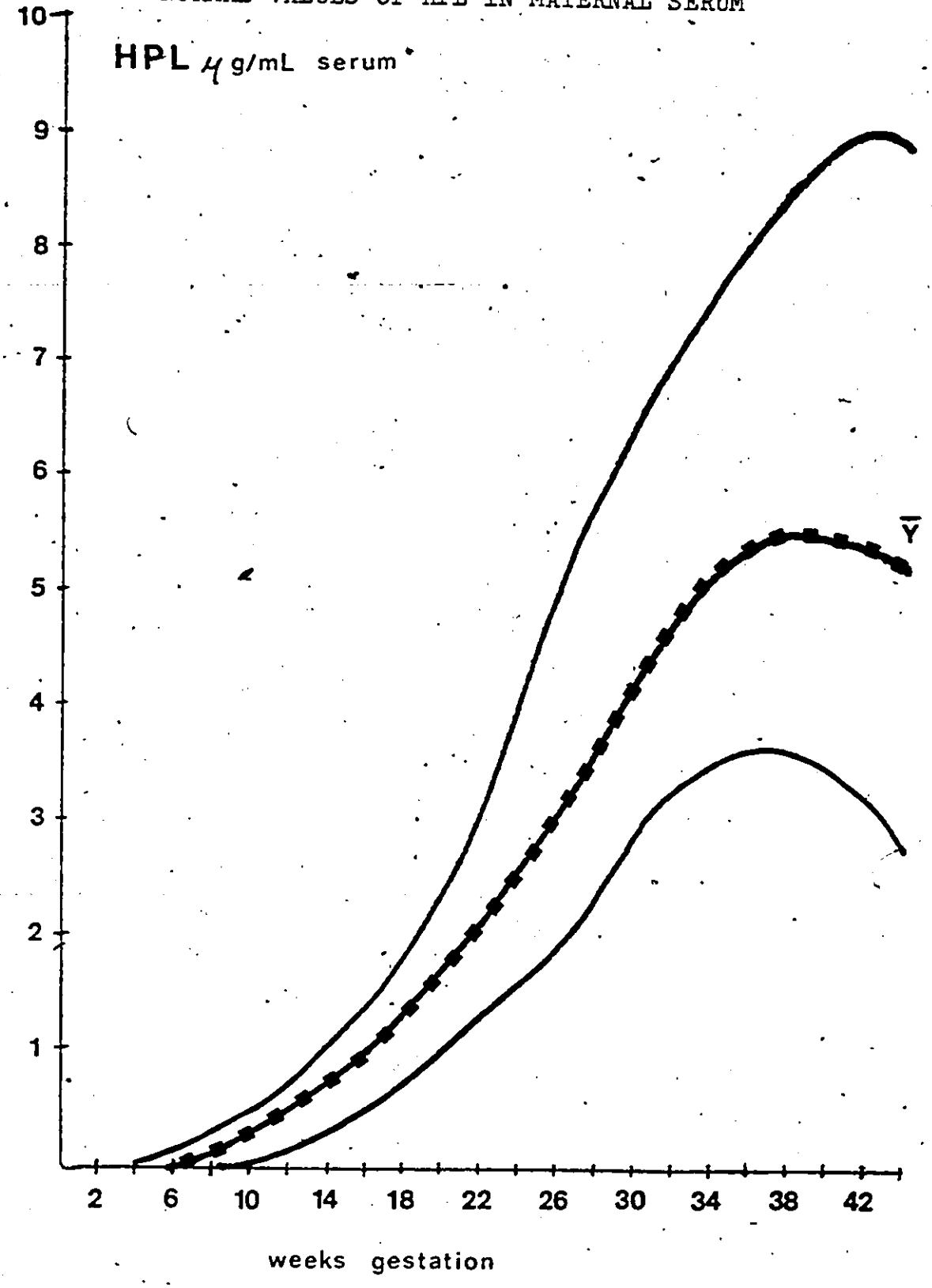
Levels are detectable in serum at about five weeks of gestation, rise to plateau at about 30 to 34 weeks, and fall slightly during the final weeks. Distribution of values is especially variable during the third trimester and is also skewed slightly toward the high end.

The broken line of the graph indicates the mean normal value ($\bar{Y}$), while solid lines show the range of variablitiy possible.

Reproduced without permission from: Phadebas hCS (hPL) Test: Clinical And Technical Information, Pharmacia Diagnostics AB, Uppsala, Sweden. (1975), p. 5.

FIGURE 10

NORMAL VALUES OF HPL IN MATERNAL SERUM



HPL reaches the fetus from the placenta. Levels in amniotic fluid are approximately 0.5 μg/mL, while cord blood contains about 0.02 μg/mL (82). The half-life of HPL has been found to be about 10 to 30 minutes (78) which makes its determination useful as an "up-to-date" measure of placental function. After delivery HPL disappears completely from the maternal circulation within a few hours.

Plasma HPL levels have been demonstrated to be independent of patient activity, food ingestion, and time of day. The absence of a circadian rhythm (83) makes sampling convenient; but irregular variations that are sometimes present (especially during the third trimester) make more than one determination necessary for proper patient evaluation.

The significance of HPL levels of maternal serum in fetal distress conditions has been debated, with some researchers claiming great value to the determinations (82,84), and others finding little or no value at all (85) or at least no more value than the conventional assays for estriol and HCG. Positive correlation, though, between placental weight and HPL values at term have definitely been established. Positive correlation with fetal weight has also been reported (86), but in other instances has been disputed. It has also been found that levels are usually, but not invariably, elevated in twin pregnancies (86).

There are several conditions in which HPL has been used to predict the existence of high-risk pregnancy and to assess fetal outcome. Of particular importance are intrauterine

bleeding with threatened abortion, pre-eclampsia, placental insufficiency, post-term pregnancies, Rh immunization, diabetes, and trophoblastic diseases. Each of these conditions will be discussed briefly.

One in every seven pregnancies ends in spontaneous abortion, although early vaginal bleeding can also occur in women who eventually deliver successfully. These two conditions must be distinguished to determine possible fetal outcome, the need for hospitalization, or the need for pregnancy termination. HPL levels have been used to predict the outcome of pregnancy in these cases of early vaginal bleeding (5 to 24 weeks). Results indicate that HPL levels are significantly lower than normal in patients who eventually abort after bleeding symptoms, while fetal survival is a likely outcome if HPL in maternal serum is normal (87).

Another condition in which HPL levels are decreased (but usually later in pregnancy) is in pre-eclampsia. Low serum HPL levels in the second and third trimester often indicate intrauterine death in the toxemic patient (84). A correlation between HPL and the severity of toxemic hypertension has also been found with higher blood pressures giving lower HPL values. This finding correlates well with the fact that the placentas of hypertensive women are often smaller than normal, undoubtedly due to the compromised circulation of the placenta present in this vasoconstrictive condition.

Placental insufficiency of any cause is invariably

associated with low HPL levels since placental size and/or function is often depressed in these conditions (86). Causes of placental insufficiency include diabetes, abruptio placentae, toxemia, placenta previa, and various unknown causes. Levels either fail to rise normally or fall abruptly during gestation. Return of values to normal often indicates a good prognosis.

Post-term pregnancies also produce a decrease in HPL levels to below normal, probably due to cessation of placental growth. Gradual falls in HPL after 40 weeks occur normally, but abrupt falls often indicate fetal distress (86).

Conditions in which maternal serum contains anti-Rh antibodies can produce elevated HPL values in maternal serum, although the determination of HPL in amniotic fluid seems to have more significance. Rh immunization accompanied by fetal hydrops, on the other hand, does produce a highly significant elevation in maternal serum HPL (82,84).

Diabetes mellitus most often produces higher than normal serum HPL levels after 28 weeks of gestation (80). This is understandable since diabetic women often deliver large fetuses, and more often than not, have very large placentas. This over-enlargement of the fetus is due to the fact that it receives excess glucose from the mother which it must deposit as excess fat and glycogen. The problem that arises in this condition is that a drop in maternal serum HPL may go unrecognized unless serial determinations are made.

In trophoblastic conditions such as hydatiform mole, and

choriocarcinoma, HPL levels are low and fail to rise normally as pregnancy continues (78). HPL determinations, then, can be used to distinguish between normal pregnancy and malignant growth since both of these conditions produce elevations in HCG.

Several RIA procedures have been used to determine HPL levels in pregnancy including ones employing double-antibody precipitation (80), dextran-coated charcoal (88), chromato-electrophoresis (89), ethanol precipitation (90), ammonium sulfate precipitation (90), and polyethylene glycol precipitation (62) methods for the separation of free and bound antigen. A solid-phase assay has also been developed (91).

Highly purified preparations of HPL (greater than 95%) are available and can be injected into rabbits, guinea pigs, or sheep using complete Freund's adjuvant to stimulate antibody production. Alternatively, HPL can be purified in the laboratory from human placentas by one of several available methods described in the literature (89). Injection of the antigen every two weeks produces antisera of high titer and sensitivity after about eight weeks. Antiserum can be diluted and frozen at -20°C and is stable for up to three years (92).

Tracer may be produced by chloramine T iodination with ^{125}I (90,92). Damaged labeled material is separated by gel filtration on Sephadex G75 to produce immunoreactivities up to about 95%. Efficiency of iodination is about 60 to 70% with about 70 µCi/µg specific activity achievable. Tracer diluted with buffer to about 250,000 CPM/mL and stored at -20°C

minimizes damage which increases to about 10 to 15% in 45 to 60 days. Use of tracer greater than 45 to 60 days old may result in decreased sensitivity of the assay.

Because of the high concentration of reactants that are usually present, the binding reaction reaches equilibrium rapidly, and incubation times of a few hours at most are required. Further reduction of incubation time to less than an hour may be achieved. Although high sensitivity is not extremely important for most purposes, assays capable of detecting 0.05 ng/mL have been developed. Most assays have sensitivities ranging from about 0.01 µg/mL to 0.1 µg/mL for use with specimens obtained during the first trimester of pregnancy (93).

One of the major problems associated with the radioimmunoassay of HPL is the lack of a suitable international reference standard (78). Although several highly purified preparations can be obtained (e.g., from NIH or NIAMDD), they may vary in exact composition and concentration. Lack of an international standard accounts for the high variability of results obtained in different laboratories and also encourages the serial monitoring of HPL for proper diagnosis.

Cross reaction of antibody with hGH has been found to be minimal (64) for two reasons: 1) the sensitivity of the antiserum is at least 1000 times greater for HPL than hGH; and 2) the concentration of HPL in pregnancy is nearly 1000-fold the concentration of hGH (80)--even in acromegalic subjects. Cross reaction of HCG up to 1000 IU/mL has also been tested, and has been shown to be negligible (94).

CHAPTER V

EXPERIMENTAL

Reagents

(A). "HPL RIA KIT" (Amersham Corp., 2636 S. Clearbrook Dr., Arlington Heights, Ill. 60005)

Tracer: lyophilized HPL labeled with ^{125}I, 150 mg sodium barbitone, and 16 mg HSA (<10 µCi ^{125}I).

Antibody: lyophilized sheep anti-HPL serum, 15 mg HSA, and buffer.

Separating agent: not supplied, Gold Shield alcohol (absolute ethanol).

Standards: lyophilized, four vials containing solids from 0.54 g serum with values when reconstituted of 1.0, 3.25, 6.15, and 10.5 µg/mL, respectively.

HPL-free serum: lyophilized, containing solids from 5.4 g serum.

Buffer: lyophilized, containing 15 mg HSA.

(B). "Phadebas hCS (hPL) Test" (Pharmacia Laboratories, 800 Centennial Ave., Piscataway, N.J.. 08854)

Tracer: lyophilized, 100 ng HPL-^{125}I (about 3 µCi ^{125}I).

Antibody: lyophilized anti-HPL raised in rabbits.

Separating agent: not supplied, Gold Shield alcohol (absolute ethanol).

Standards: lyophilized, five vials containing 1.0, 2.0, 4.0, 8.0, and 16.0 µg/mL HPL, respectively, after reconstitution.

HPL-free serum: lyophilized diluted horse serum.

Buffer: 1.06 g powdered components, pH 7.4 after reconstitution.

(C). "Human Placental Lactogen Kit" (Cambridge Nuclear, 575 Middlesex Turnpike, Billeruca, Mass. 01865)

Tracer: lyophilized HPL-^{125}I ($\geqslant$5μCi ^{125}I).

Antibody: lyophilized sheep anti-HPL serum.

Separating agent: lyophilized goat or rabbit anti-sheep gamma globulin.

Standards: lyophilized, six vials containing 1.0, 0.5, 0.25, 0.1, 0.05, and 0.025 μg HPL, respectively, in 500 μL of human serum. Values after reconstitution: 2.0, 1.0, 0.5, 0.2, 0.1, and 0.05 μg/mL, respectively.

HPL-free serum: lyophilized human serum.

Buffer: lyophilized, 1% BSA in 0.1 M borate, pH 8.5 after reconstitution.

Normal sheep serum: lyophilized serum used to reconstitute tracer.

Controls: Ortho RIA Control Serum III and IV (Ortho Diagnostics, Raritan, N.J. 08869); lot #'s 10R603 and 11R703.

Miscellaneous Reagents:

Sephadex G75 lot #1866 (Pharmacia Fine Chemicals, Piscataway, N.J. 08854)

Dulbeccos PBS lot #R968305 (Grand Island Biological Co., New York, N.Y.)

Blue Dextran 200 lot #4409 (Pharmacia Fine Chemicals, Piscataway, N.J. 08854)

Borate Buffer (0.113 M): 16.5 g boric acid, 5.4 g NaOH, 20 g BSA, 10 mL 10% sodium azide, g.s. 2000 mL, pH 8.5.
Purified HPL: lyophilized 5 mg HPL, potency estimate 95% (gift from National Institute of Arthritis, Metabolism, And Digestive Diseases (NIAMDD), Bethesda, Md.).

Equipment

All pipetting was performed with precalibrated SMI or glass volumetric pipettes. Polystyrene 12 x 75 mm tubes with plastic caps were used. Tubes were centrifuged at 4°C at 1500xg for at least ten minutes on an IEC PR-6000 refrigerated centrifuge (International Equipment Co., Needham Heights, Ma. 02194). Counting was done on a Searle Analytic Model 1285 Automatic Gamma System (Searle Analytic, Des Plaines, Ill. 60018) equipped with a Western Union paper tape teletype and printer. Data was processed on a Hewlett Packard 9831 computer with tape reader and printer using RIA software package #09831-14254 (Hewlett Packard, Loveland, Co. 80537) (see APPENDIX D for calculation methods). Chromatography was performed using a 100-mL glass buret (1.5 cm diameter), and fractions were collected with a Gilson FC-100K microfractionator (Gilson Medical Electronics, Middleton, Wis.).

Procedures

Manufacturer's procedures for the determination of HPL

during the third trimester (third trimester standard curves) were followed according to the package insert, and samples were run at least in duplicate. For the tracer displacement studies, lyophilized tracer was reconstituted with less than the usual amount of buffer to obtain the desired concentrations (e.g., 2x, 4x, etc. of the normal amount of tracer). For the immunoreactive studies, a similar procedure was followed to produce more concentrated antibody solutions. Using the Cambridge Nuclear kit, the procedure was slightly modified to insure adequate precipitation by the second antibody. Three times the normal amount of precipitating antibody (as recommended by the manufacturer) was added to each tube by using a more concentrated solution of this reagent. Also, the second incubation time was increased from ten minutes to 1½ hours--until a visible separation of layers could be seen. Centrifugation and decantation was carried out as usual.

Chromatography of tracer was performed using Sephadex G75. The gel was prepared in phosphate buffered saline (PBS) (pH 7.4) and allowed to stand at room temperature for three days. The slurry was deaerated under vacuum, and a 56-cm column was poured. The column was equilibrated with 0.113 M borate buffer (pH 8.5) containing 1% BSA. Lyophilized tracer was diluted with borate buffer to a concentration ten times that normally used in the assay, and a small amount of Blue Dextran was added as a marker. Tracer was added to the column and eluted in 0.5-mL fractions at a flow rate of about 23 mL/hr.

Void volumne of the column was approximately 14 mL. Appropriate fractions were pooled, added to lyophilized normal sheep serum (NSS), and the final volume brought up to 10 mL.

Cross reaction studies were done using aqueous solutions of hGH and HCG with concentrations of 50 ng/mL and 10 IU/mL, respectively. Two-fold serial dilutions were made of these solutions using HPL-free serum to yield concentrations of hGH from 50 to 3.13 ng/mL and HCG from 10 to 0.625 IU/mL. Twenty microliter samples of these dilutions were run as unknowns in the assay. Since a 1/10 dilution is normally made on specimens before assay, the above dilutions corresponded to serum values of 500 to 31.3 ng/mL hGH and 100 to 6.25 IU/mL HCG.

Recovery studies were performed using purified HPL obtained from NIAMDD. The vial containing 5 mg was reconstituted with 5 mL PBS to yield a concentration of 1 mg/mL. Twenty microliters of this solution was added to 1 mL of HPL-free serum from which 20 µL had been removed. Two-fold serial dilutions were made using HPL-free serum to produce concentrations ranging from 20 µg/mL to 0.156 µg/mL. Samples with concentrations above 1.25 µg/mL were further diluted 1/10 with HPL-free serum so that they could be assayed using the Cambridge Nuclear standard curve. Samples with concentrations below this value were run undiluted.

CHAPTER VI

RESULTS AND DISCUSSION

Sensitivity

Sensitivities of the methods determined by multiple assay of the zero tube are given in Table I. Mean CPM's were calculated for the B_0 tube, and this value minus 2s or minus 10% gave corresponding sensitivities as concentrations read from the standard curves. For all three kits evaluated, calculation using the 95% confidence interval gave better sensitivities than use of mean minus 10%.

Sensitivity was also assessed as least detectable dose by dilution of the lowest standard. These values generally were lower than the above determined sensitivity levels indicating that sensitivity was limited by the precision near zero dose (see Table I). It should be mentioned that all values were applicable to the third trimester standard curves, and that assay sensitivity for Amersham (A) and Pharmacia (P) could be improved by dilution of antibody and use of lower concentrations of standards. Cambridge Nuclear (CN) utilizes only one standard curve for all three trimesters, and instead dilutes second and third trimester patients 1/10 in order to use a standard curve ranging in values from 0.05 to 2.0 µg/mL. In this case it was assumed that patients would be diluted, so that actual sensitivity was multiplied by a factor of ten to compensate for this. The actual sensitivities

TABLE I

SENSITIVITIES OF HPL KITS

	A	CN	P
Zero dose -2s (n = 5)	0.3 µg/mL	0.1 µg/mL	0.6 µg/mL
Zero dose -10% (n = 5)	1.4 µg/mL	0.6 µg/mL	1.5 µg/mL
Standard dilution	0.25 µg/mL	0.13 µg/mL	0.25 µg/mL
Slope of B/F vs. dose	-0.416	-3.12	-0.618

were taken as the value calculated from precision of the zero tube since this value was higher than that determined by dilution of the lowest standard.

If sensitivity is considered as by Yalow (11) to be the slope of the dose response curve near zero dose, it is seen that CN has the highest theoretical sensitivity with P next and A lowest. The reason for the low actual sensitivity of P is due to the poor precision of the zero standard. This further proves that sensitivity depends both on the slope of the dose response curve and on the variance in the determination of the dose response variable.

Tracer Evaluation

Tracer was evaluated by three parameters: non-specific binding; immunoreactivity by addition of excess antibody; and specific activity. NSB's were determined by eliminating the primary antibody from an assay tube of zero standard. Calculation of NSB as percent of the B_0 tube ($Blk/B_0 \times 100$) on kits between one and two months before expiration yielded results shown in Table II. An assay performed with P one day subsequent to the above mentioned run resulted in a value of 31% for NSB. Several different runs performed using CN kits with tracer of varying ages (from date of iodination to week before expiration) produced NSB's ranging from 5.1% to 9.3%. The discrepancy of NSB's between kits, particularly the high result obtained from P, can possibly be explained by looking at

TABLE II

SUMMARY OF TRACER CHARACTERISTICS

	A	CN	P
NSB (as % of B_0)	8%	12%	23%
Immunoreactivity (antibody excess)	78.2%	77.4%	65%
Immunoreactive mass (tracer displacement)	--	3 ng/mL (0.3 ng/tube)	--
Manufacturer's specific activity	63.3 µCi/µg	60 µCi/µg	30 µCi/µg
Immunoreactive mass (calc. from S.A.)	2.55 ng/mL (1.3 ng/tube)	11 ng/mL (1.1 ng/tube)	2.3 ng/mL (0.5 ng/tube)

the stabilities of the kits claimed by the manufacturers.

Amersham states that their kits are stable four to eight weeks from date of receipt in the laboratory, while P claims four months and CN six weeks. The literature states that life of iodinated HPL stored at 4°C is about four weeks, while that stored at -20°C or -70°C is approximately six weeks (90). Assuming that tracer has been labeled immediately prior to receipt of the kit, a kit one month from expiration would mean that the tracer was four weeks old in the case of A, three months old in the case of P, and only two weeks old for CN. The tracer producing such a high NSB in P, then, probably was damaged because of age. This theory is supported if we look at the Scatchard plot performed using this tracer (see below).

Non-specific binding using patient sera showed no difference from that using zero standard for all three kits, indicating that use of the NSB value of the "blank" was legitimate.

Immunoreactivities of tracers were performed on kits the same age as mentioned above. In this case, excess antibody was added to bind all bindable tracer, and immunoreactivity was calculated as: $(B_0 - Blk)/T \times 100$ (see Table II). For CN, excess second antibody was added to insure complete precipitation of the antigen-antibody complex. Assuming that all non-bindable radioactivity was due to damaged tracer, kit A showed about 22% damage, P about 35% damage, and CN approximately 23%. The low immunoreactivity of P also supports the conclusion of a high amount of damaged radioactive ligand

present in the trace reagent. To see if non-immunoreactive radioactivity could be separated from immunoreactive ligand, and to see if this activity was free ^{125}I, the CN tracer was chromatographed using gel filtration (see below).

Determination of immunoreactive mass of the tracer was attempted by adding increasing aliquots of tracer according to the procedure given in APPENDIX A. For kit A there was no displacement of tracer for aliquots up to four times normal. It was assumed that mass was too small to be measured by use of the third trimester standard curve. Tracer displacement studies with CN yielded an immunoreactive mass of 3 ng/mL or 0.3 ng tracer added per tube (Table II). Tracer was not displaced with P, and again it was assumed that mass was too small to be detected using the standard curve.

Specific activities at iodination were obtained from the manufacturers for all three kits, and immunoreactive masses were calculated from these values using the formula given in APPENDIX B. These values were used when calculating Scatchard plots (see below). The discrepancy seen in the CN experimental vs. calculated value (3 ng/mL vs. 11 ng/mL) was attributed to decay of tracer and to experimental error in measuring tracer mass.

Antibody Characteristics

Scatchard plots were graphed for all three kits using data obtained from standard curves by use of the procedure

given in APPENDIX B. When using the method of correcting for non-specific binding as recommended by Chamness (54), and as given in APPENDIX B, it was found that the CN kit showed a backbending of the plot at standard values greater than 0.5 µg/mL. Pharmacia also showed a backbending, but of such severity, that mass bound for standards with concentrations greater than 4.0 µg/mL was calculated as less than zero. This observation raises the question of whether this NSB correction is necessary, or even actually justified. Several arguments against use of this correction can be formed. First of all, Chamness assumes the presence of a low-affinty, high-capacity binder in the system as evidenced by a long "tail" on the uncorrected Scatchard plot. This may not be the case in all assays. Also, the assumption that Ag^* binds non-specifically to the same extent as Ag in the standards may not be true, especially if damaged labeled ligand is present. If Ag^* binds non-specifically to a greater extent than Ag, then calculation of NSB of total antigen will be much overestimated if the formula $F(\frac{Blk-Bkg}{T_c-Blk})$ is used. It is also true that NSB may be different in the presence of antibody, and in fact, is usually lower. Thus, use of an NSB tube without antibody tends to overestimate this value. Because of these reasons, I have eliminated the NSB correction from the calculation of Scatchard plots during this evaluation, and I feel that the issue needs further study before the correction is routinely used.

Scatchard plots for the three kits can be seen in figure 11.

FIGURE 11

EXPERIMENTAL SCATCHARD PLOTS

Legend

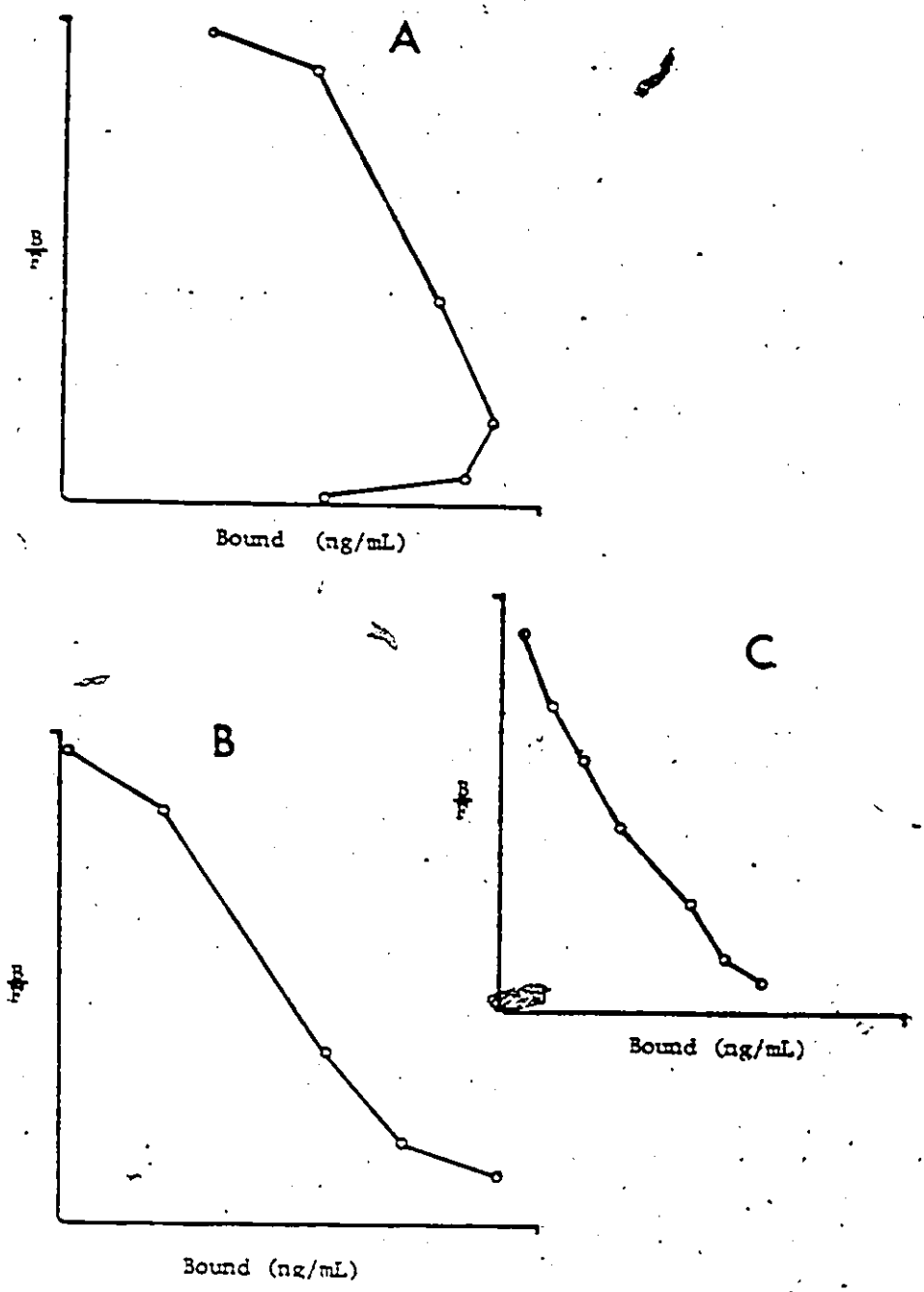
A. Typical plot of P shows backbending due to either tracer damage or non-equilibrium conditions.

B. This plot of A showed the presence of multiple binding sites (heterogeneous antiserum). The flattening of the curve at low bound mass values was probably due to tracer damage which will produce a greater change in B/F than B at low analyte concentrations.

C. Cambridge Nuclear also showed the common heterogeneous antiserum plot.

FIGURE 11

EXPERIMENTAL SCATCHARD PLOTS



Kits A and CN showed typical plots indicating heterogeneous antisera. The slight flattening of the curve of kit A (Fig. 11B) between zero and the 1.0 µg/mL standard could be due to the presence of damaged tracer. Mass bound is calculated as B/T(mass tracer + mass standard), and since a decrease in B/T from damaged tracer is greater at low analyte concentrations, the calculated mass bound will be lower than expected. The B/F ratio will also be lower than expected, and to an even greater extent than B since B/F is even more greatly influenced if B/T decreases. Pharmacia showed considerable backbending at standard values greater than 4.0 µg/mL. Again, this may be an indication of tracer damage, or may also be due to non-equilibrium conditions.

Scatchard plots run with two times and one half the normal concentration of antibody are shown in figures 12 to 14. All three kits showed parallelism of curves (within experimental error) upon changing antibody concentrations. This fact helps to confirm equilibrium constants calculated from slopes of the Scatchards. The curve obtained from CN (Fig. 14) using 2x antibody shows a flattening at low bound mass. This could be due to damaged tracer, as indicated above, or may be due to the phenomenon of positive cooperativity. Confirmation is necessary, though, by repeat analysis with lower standard concentrations to verify a maximum in the curve.

Equilibrium constants calculated from the average slopes of the normal Scatchards are given in Table III, along with x-intercepts which show the total concentration of antibody.

FIGURES 12, 13, 14

SCATCHARD PLOTS WITH VARYING ANTIBODY CONCENTRATIONS

Legend

Assays were run with the normal amount of antiserum; and the three lowest standards were also run using one half (½ab) and two times (2ab) this amount of antiserum. Only the two lowest standards were run with 2ab in the P kit. All kits show that a change in antibody concentration does not change the slope of the Scatchard plot. Thus, the equilibrium constant calculated from the normal Scatchard slope is most probably accurate.

Fig. 12: Amersham Scatchard plots with differing antibody concentrations showed relatively good parallelism.

Fig. 13: The backbending of the Scatchard plot of Pharmacia was repeated with ½ antibody concentration.

Fig. 14: Cambridge Nuclear also showed good paralellism of Scatchard plots, although the slight flattening of the curve of 2ab may indicate a positive cooperativity effect.

FIGURE 12

AMERSHAM SCATCHARD PLOTS

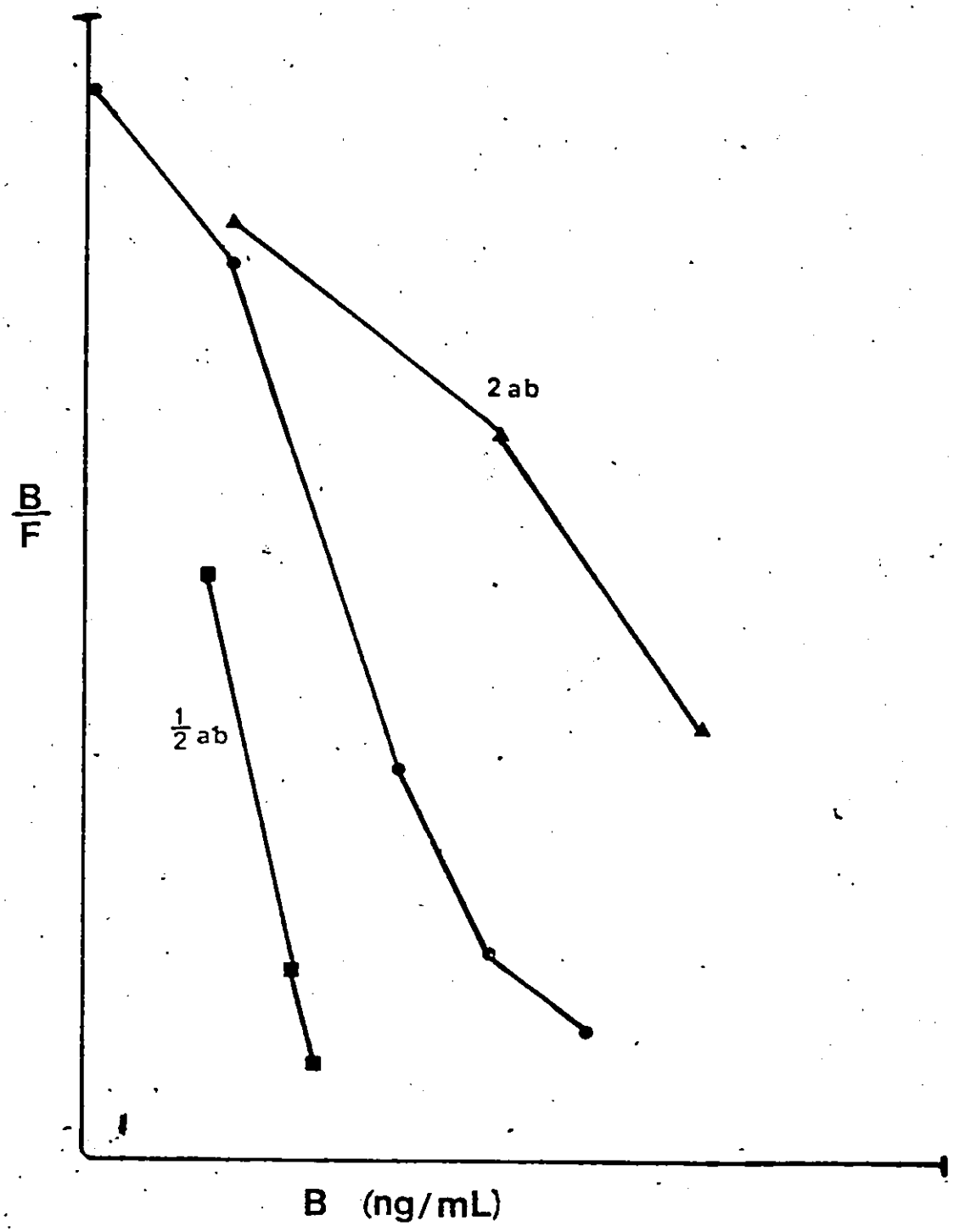


FIGURE 13

PHARMACIA SCATCHARD PLOTS

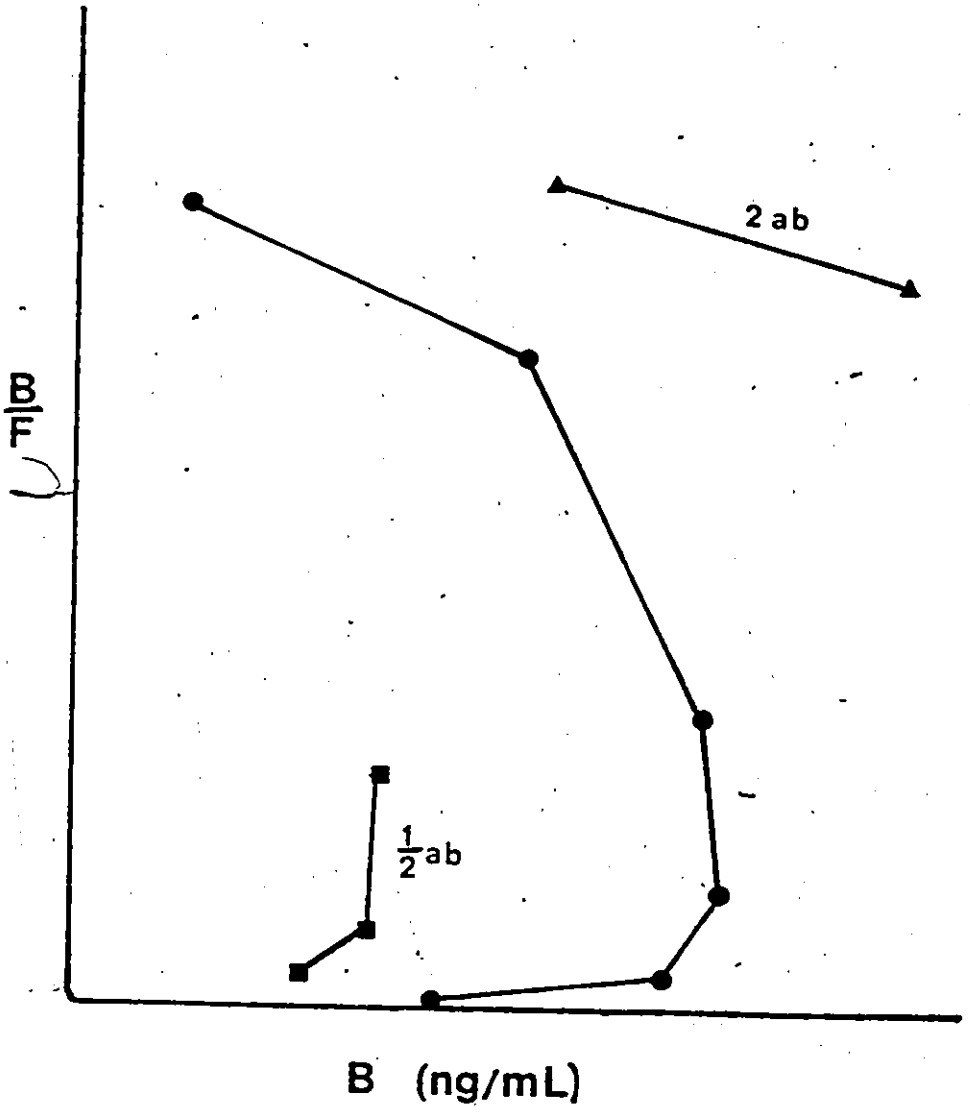


FIGURE 14

CAMBRIDGE NUCLEAR SCATCHARD PLOTS

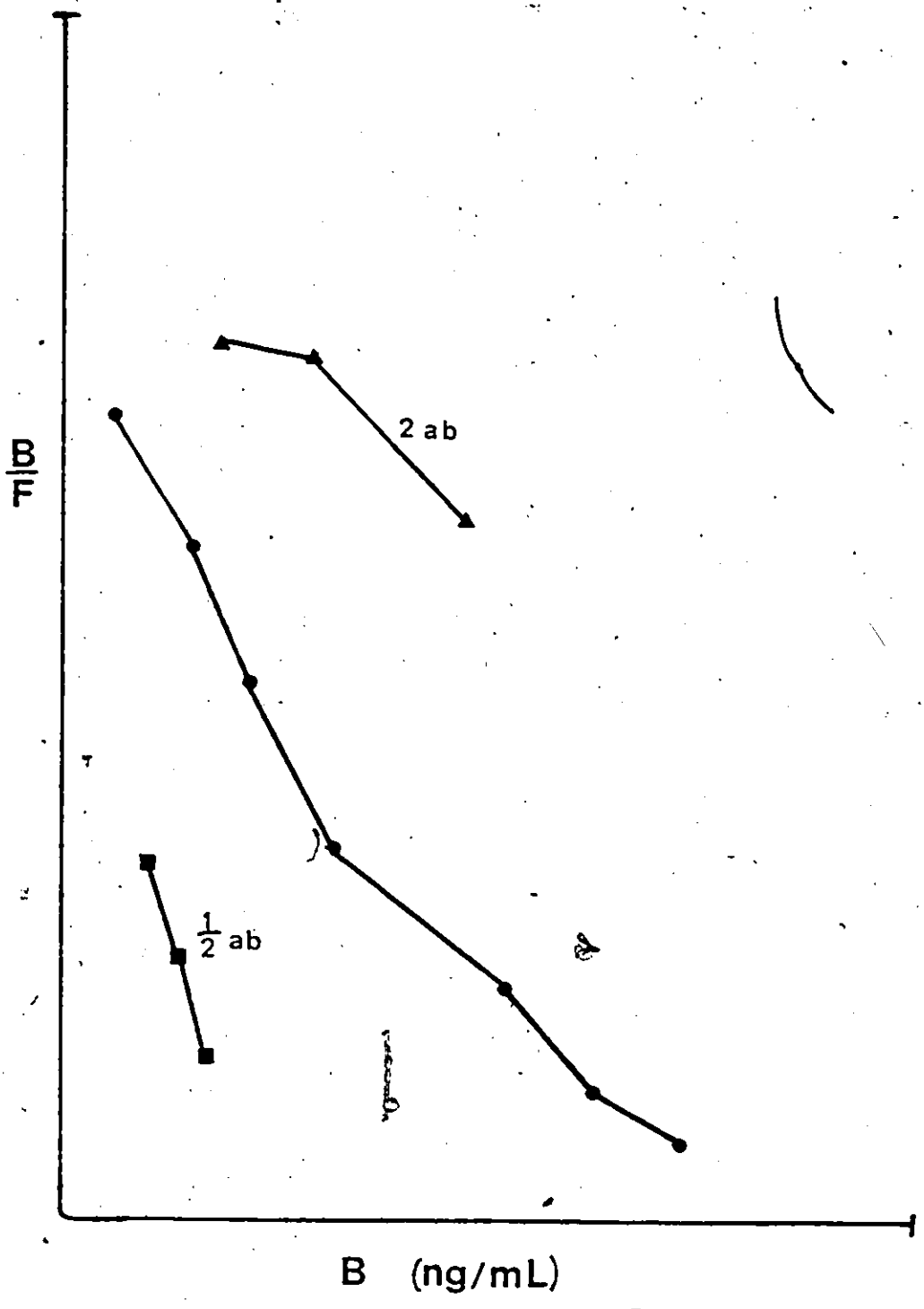


TABLE III

SUMMARY OF ANTIBODY CHARACTERISTICS

	A	CN	P
K_{eq}	6.13×10^8 L/mol	1.62×10^9 L/mol	5.31×10^8 L/mol
Ab^0	0.13 μg/mL	0.016 μg/mL	0.26 μg/mL
Scatchard plots	Satisfactory	Satisfactory	Unsatisfactory (backbending)

binding sites. Slope of P was calculated only on points below 4.0 µg/mL, i.e., before backbending of the curve began. As can be seen, CN has the antiserum with the lowest concentration of binding sites and the highest equilibrium constant. This is probably the reason for the greater sensitivity of the kit.

Parallelism Studies

Results of parallel studies using a high dose patient are shown in figures 15 to 17. All three kits showed patient curves which did not differ significantly from the standard curves. This fact indicates probable similarity in standard and unknown reactions with antibody, and also indicates that a dilution of the patient can be used if necessary in the assays. A further check on the CN kit was performed since both diluted and undiluted samples are routinely assayed together, depending on the trimester of pregnancy. Two samples with approximately 1.0 µg/mL and 2.0 µg/mL concentrations were run both undiluted and diluted 1/10 in HPL-free serum. The diluted result was then multiplied by ten and compared to the result obtained on whole serum. Results were very favorable: diluted, 1.33 and 2.48 µg/mL versus undiluted, 1.39 and 2.48 µg/mL.

Precision

Results of precision studies using Ortho controls are

FIGURES 15, 16, 17
PARALLELISM STUDIES

Legend

Figures show the standard curves (solid lines) versus the curves obtained by dilution of a high-dose patient with HPL-free serum (dashed lines). The value of the undiluted sample was obtained by interpolation from the standard curve, and may account for the slight deviations of the A and P curves.

Fig. 15: Amersham showed a small but insignificant deviation toward low dose levels.

Fig. 16: Pharmacia showed relatively good parallelism. The value at about 1.0 µg/mL was approaching the limit of sensitivity and may be in error.

Fig. 17: Cambridge Nuclear showed very good parallelism of patient dilutions.

FIGURE 15

AMERSHAM PARALLELISM

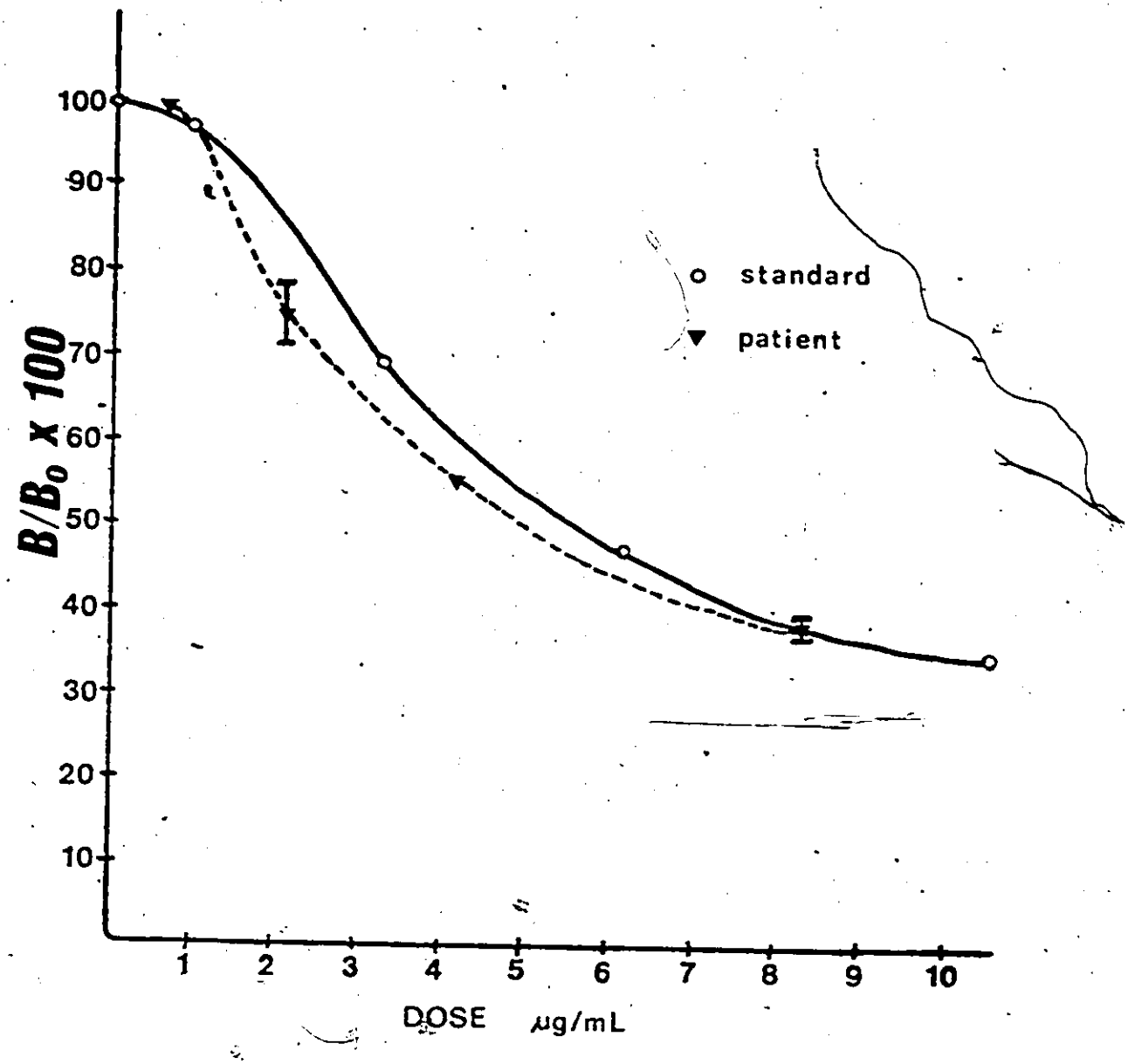


FIGURE 16

PHARMACIA PARALLELISM

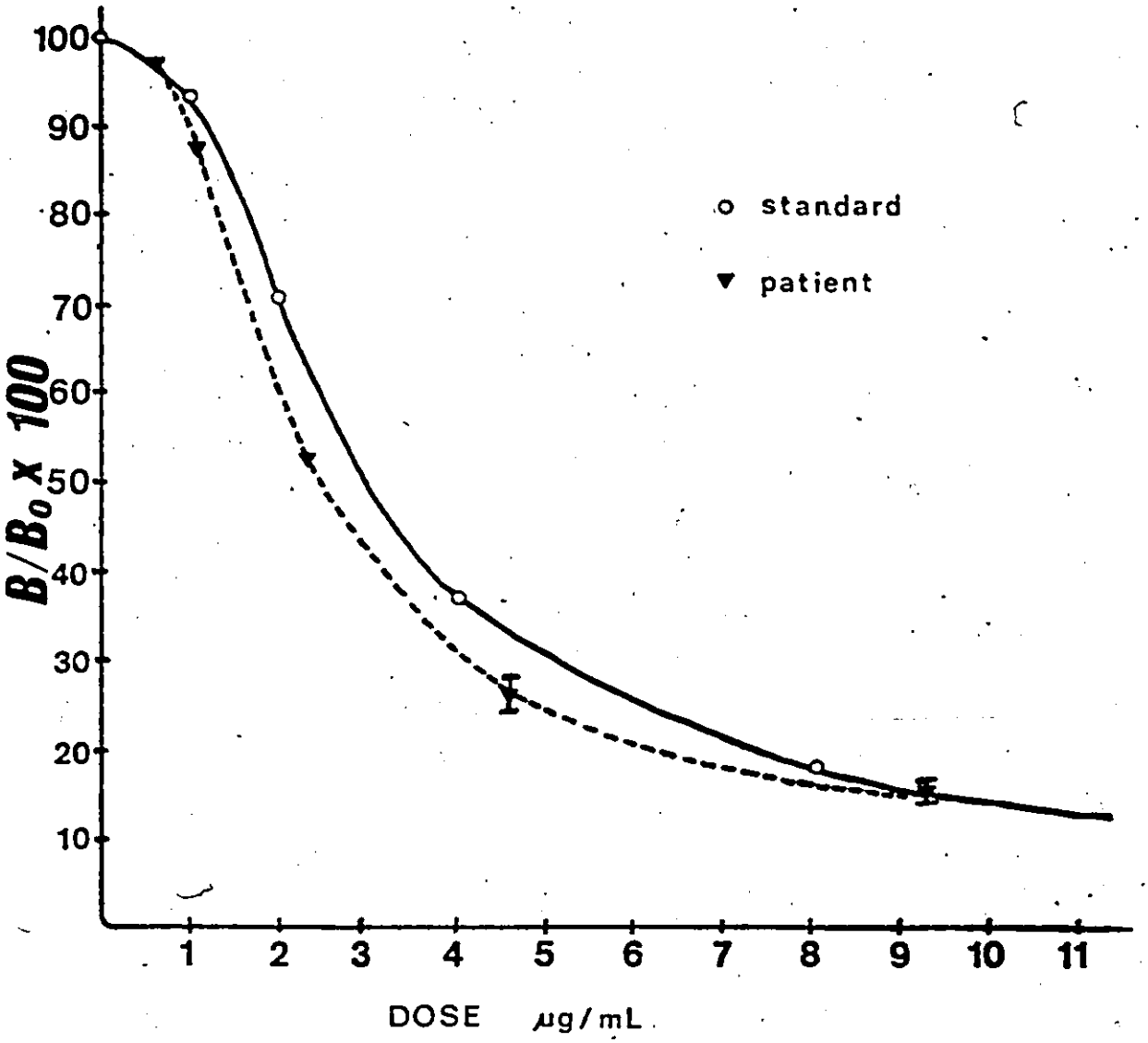
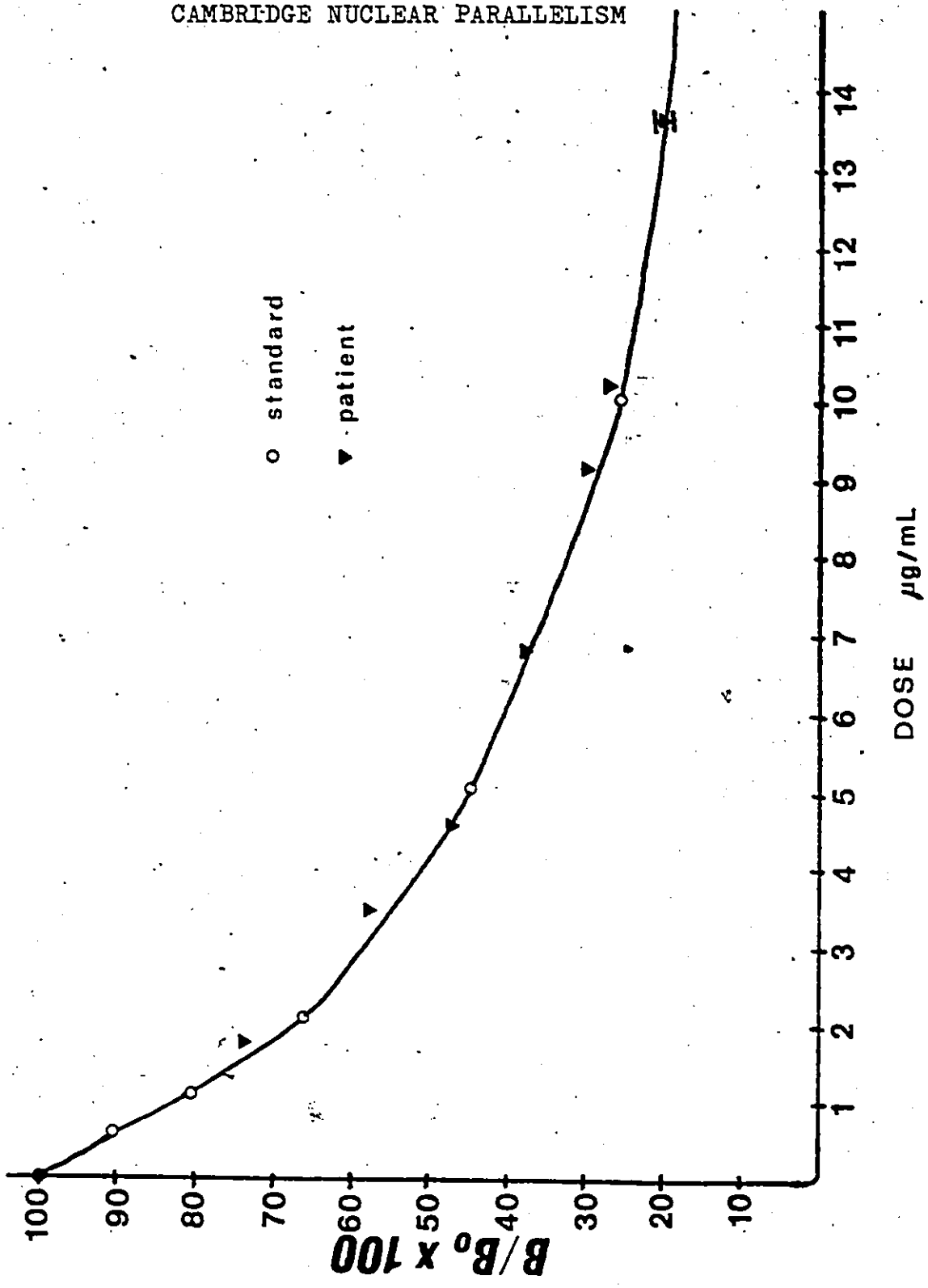


FIGURE 17

CAMBRIDGE NUCLEAR PARALLELISM



seen in Table IV. Inter-assay results were calculated from values obtained after duplicate assay of controls, and using the weighted Rodbard transformation of data (APPENDIX D). Although n was not large enough to give statistically significant results, a general idea of assay precision could be formulated. It was found that the C.V.'s obtained agreed relatively well with the claims of other researchers developing or evaluating HPL assays (90,92,95).

Intra-assay precision was determined as the precision (C.V.) between duplicates as calculated by the computer using the formula given in APPENDIX D. Again, although results cannot be statistically evaluated, an idea about intra-assay precision can be obtained. The consistently poor precision of P may again be explained by the presence of a large amount of damaged tracer molecules.

Observation of the Dose Response Curves

Standard curves were manually plotted in several ways including: B/F *vs.* dose, 1/B *vs.* dose, B/B_0 *vs.* dose, and B/T *vs.* log dose. Typical B/F *vs.* dose curves are seen in figure 18. For A, a slight flattening of the standard curve below the 1.0 µg/mL standard was observed in all of the plots, particularly B/B_0 vs. dose HPL. The same was true for P, although to not as great an extent. This may partially account for the poorer sensitivities of these assays compared to CN. Both CN and P showed flattening of the B/F curves at higher

TABLE IV

PRECISION COMPARISONS

	A		CN		P	
Inter-assay →	OIII	OIV	OIII	OIV	OIII	OIV
n	4	4	8	8	2	2
$\bar{x}$	3.63	7.18	3.62	8.50	4.19	8.27
s	0.39	0.19	0.33	0.57	0.26	0.66
C.V.	10.7%	2.7%	9.0%	6.7%	6.2%	8.0%
Intra-assay						
n	3	3	7	7	2	2
C.V.	2.67%	0.77%	1.07%	1.51%	7.45%	10.8%

Ortho lot #'s:

OIII: 10R603

OIV: 11R703

FIGURE 18

COMPARISON OF DOSE RESPONSE CURVES (B/F vs. DOSE)

Legend

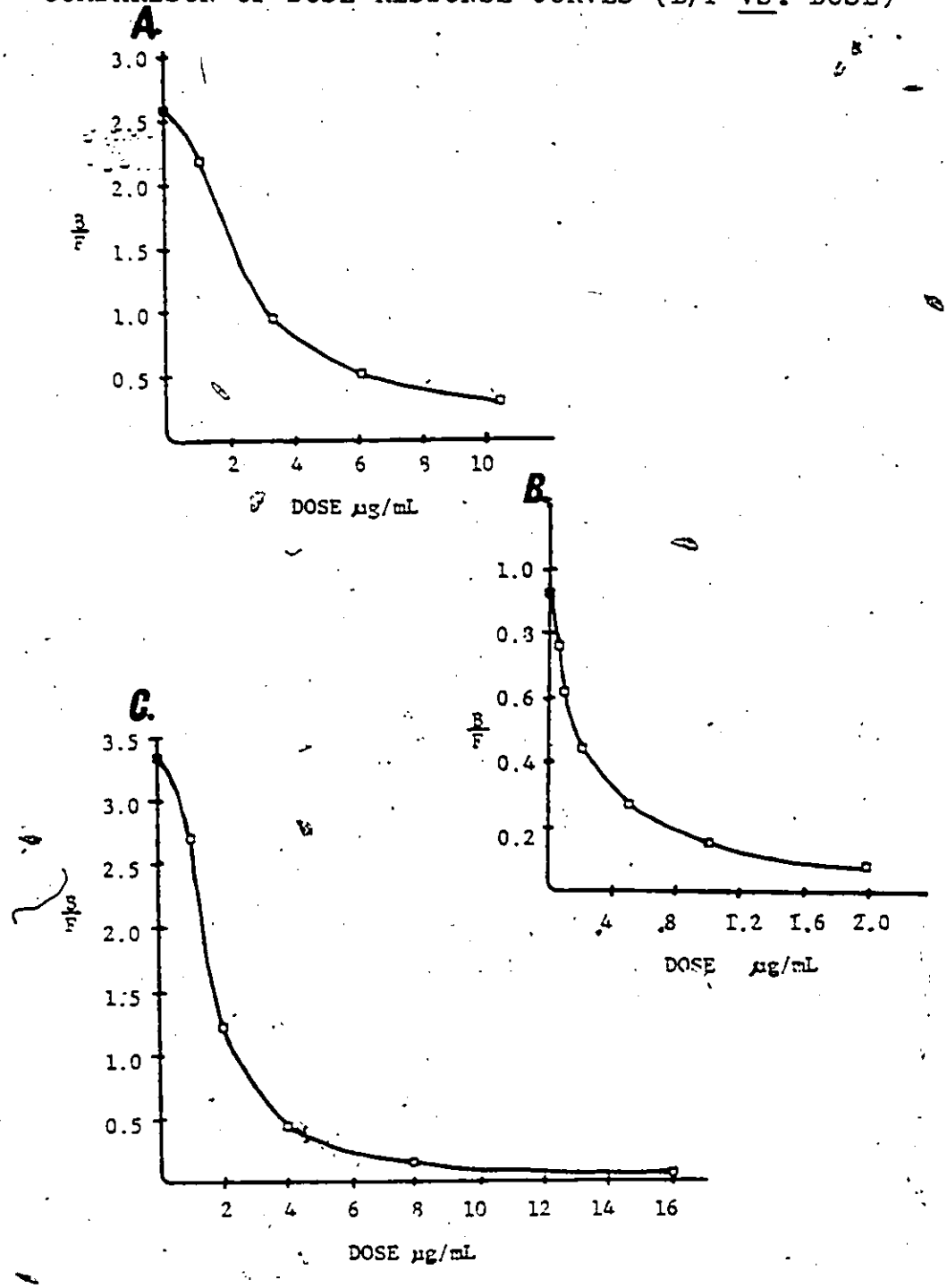
A. Amersham showed a flattening of the curve below 1.0 µg/mL, but had satisfactory resolution at the higher end of the curve.

B. The CN plot of B/F vs. dose HPL indicated good sensitivity and resolution at low concentrations, but a flattening of the curve, and thus poorer resolution at the higher end of the curve.

C. Pharmacia had very poor resolution at doses greater than 8.0 µg/mL, and also a flattening of the curve below 1.0 µg/mL.

FIGURE 18

COMPARISON OF DOSE RESPONSE CURVES (B/F VS. DOSE)



dose levels indicating decreased resolution at levels greater than about 10 µg/mL for CN and 8 µg/mL for P.

Logit-log plots of P and A showed that a hyperbolic fit of the data may be more appropriate than the linear fit (Fig. 19). This fact may account for a discrepancy in the value for Ortho III control depending on the method used to plot the standards for kit A. The concentration for this control obtained from manual plots was about 3.2 µg/mL, while that obtained with the weighted and unweighted Rodbard plots was 3.8 µg/mL. In both P and CN both manual and computer plots yielded indistinguishable results.

Accuracy

The accuracy of CN was evaluated by use of a purified preparation of HPL, and by split-sample comparison against a procedure routinely used by another laboratory. Recovery data obtained by use of purified HPL additions to HPL-free serum is shown in Table V and figure 20. Although a proportional error is indicated by the results, for statistically significant conclusions the experiment should be repeated several times with different dilutions made to elimipate pipetting erros. Recovery of HPL procedures as stated in the literature range from about 96% to 107% (92).

Split-sample analysis gave results summarized in figure 21. Again, a positive proportional error for CN is indicated, along with an absolute error as determined from the

FIGURE 19

COMPARISON OF DOSE RESPONSE CURVES (LOGIT-LOG PLOTS)

Legend

Both Amersham (curve A) and Pharmacia (curve C) produced hyperbolic logit-log transformations, while a linear fit was satisfactory for Cambridge Nuclear (curve B). A discrepancy between manually plotted and computer logit-log dose interpolation was found only with the Amersham kit.

FIGURE 19

COMPARISON OF DOSE RESPONSE CURVES (LOGIT-LOG PLOTS)

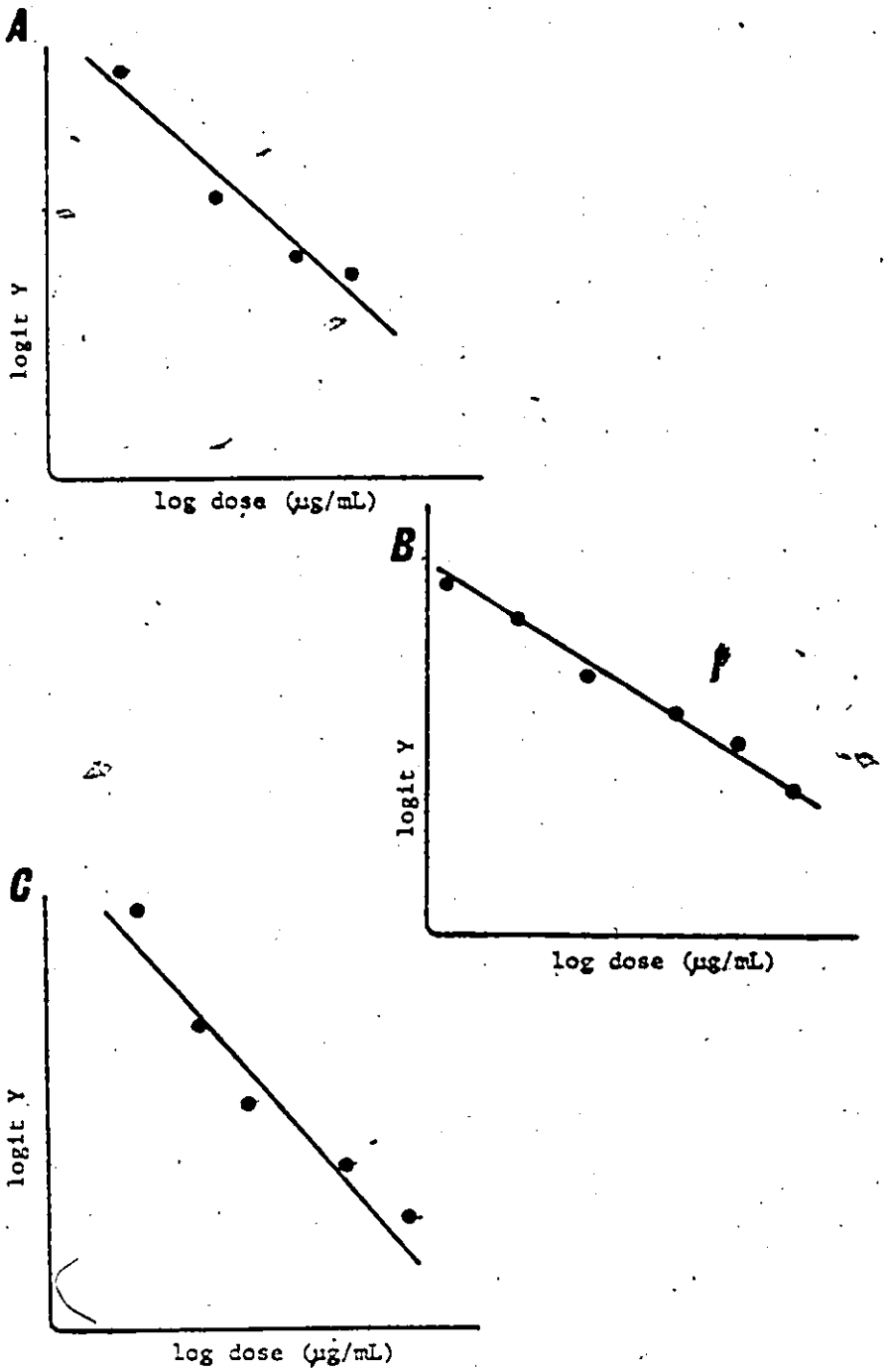


TABLE V

RECOVERY DATA

Expected	Obtained	95% range	Recovery
20.0 µg/mL	23.84	20.52 - 28.21	119%
10.0	11.57	10.55 - 12.75	116%
5.0	5.36	4.95 - 5.82	107%
2.5	2.88	2.77 - 2.99	115%
1.25	1.58	1.47 - 1.71	126%
0.625	0.78	0.75 - 0.82	125%
0.313	0.38	0.34 - 0.42	121%
0.156	0.20	0.18 - 0.22	128%

FIGURE 20

RECOVERY OF PURIFIED HPL

Legend

Expected results plotted against obtained results indicated that a positive proportional error was present. The low y-intercept confirmed the assumption that the base was actually "HPL-free" serum, while a correlation coefficient close to unity supported a linear relationship between the expected and observed results.

FIGURE 20

RECOVERY OF PURIFIED HPL

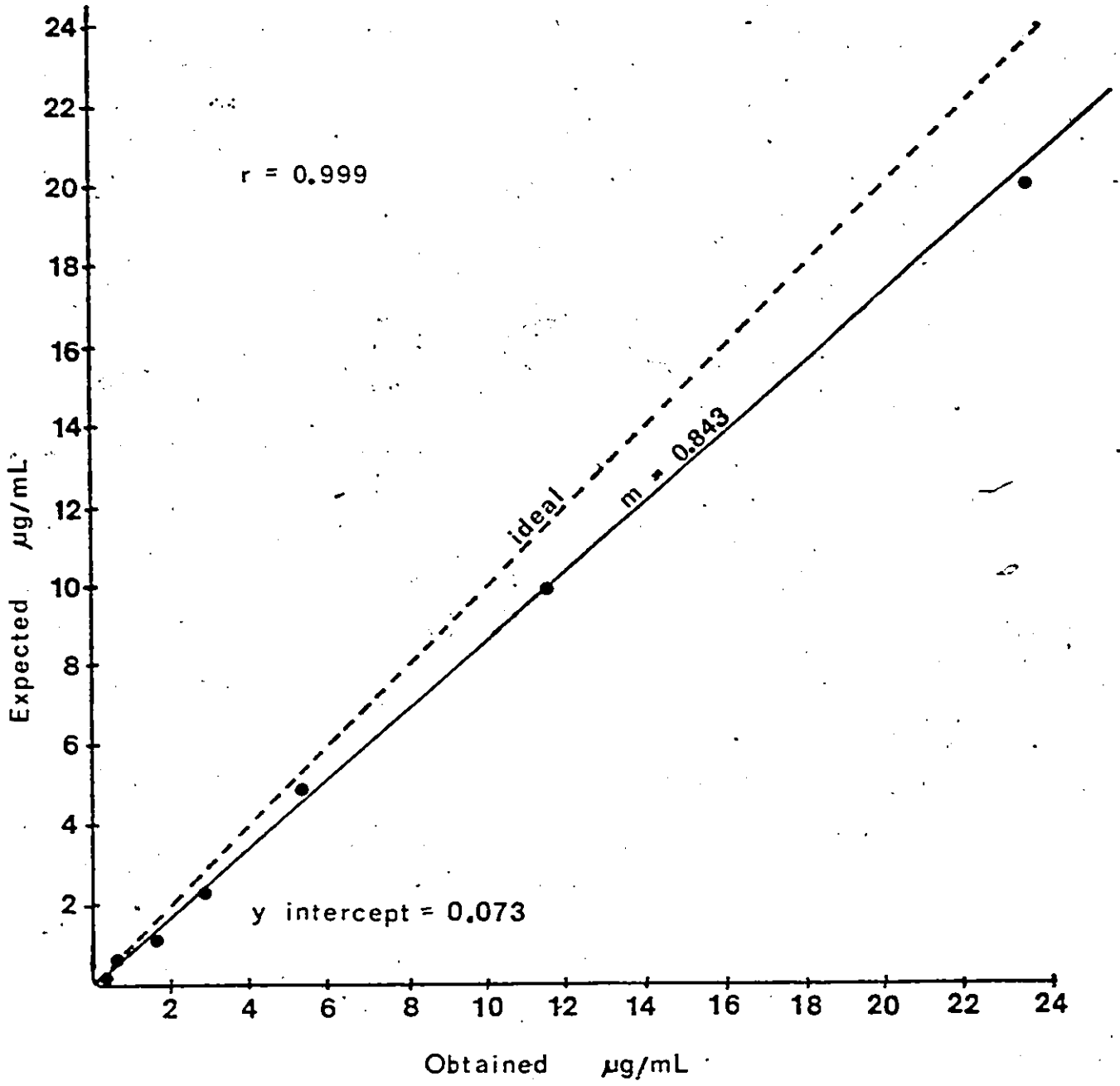


FIGURE 21

SPLIT-SAMPLE ANALYSIS

Legend

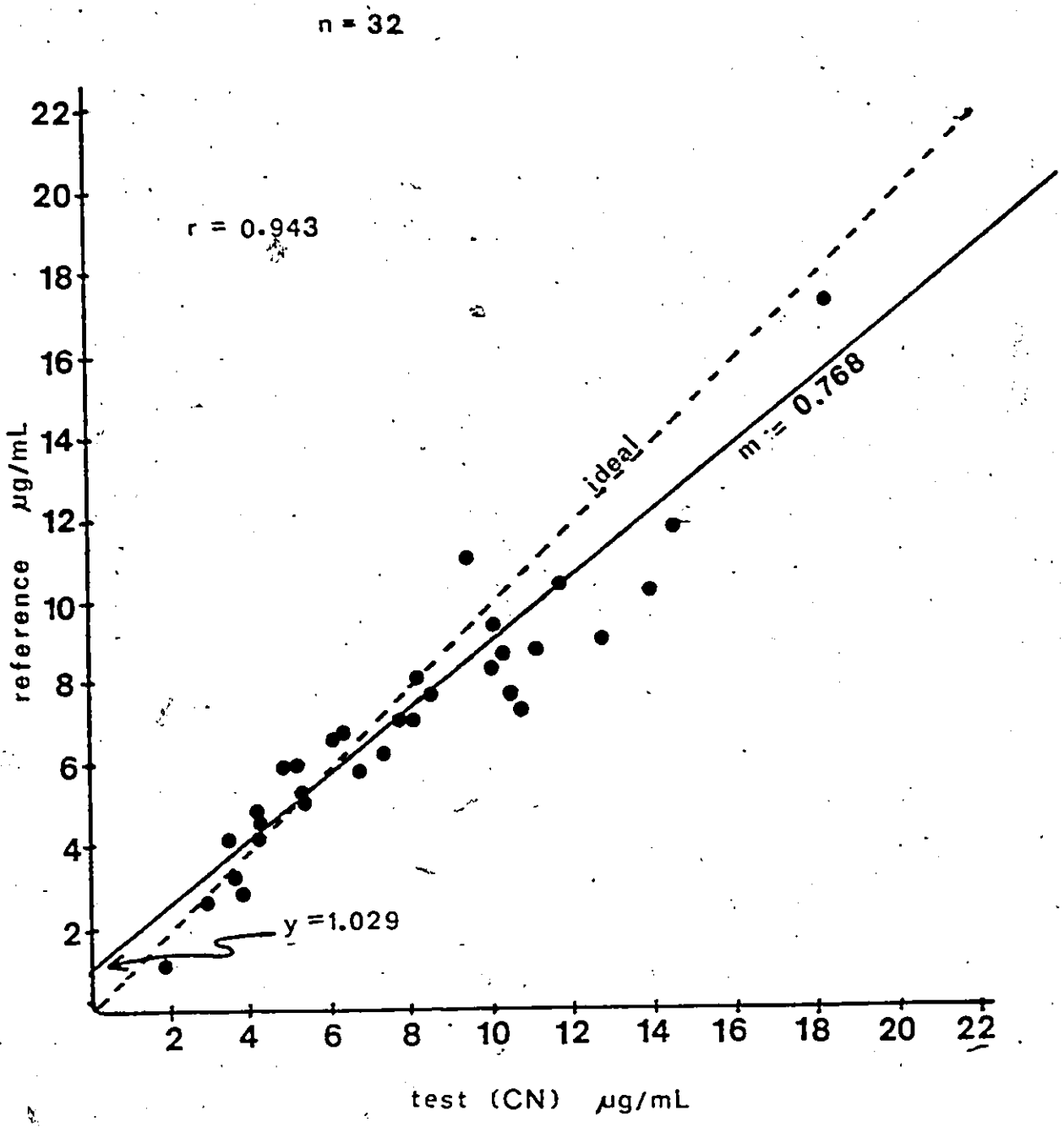
If all data points are considered, it appears that the test procedure (CN) has a positive proportional and absolute error compared to the reference procedure. If the curve is considered in two parts, i.e., points less than 8.0 µg/mL, and points greater than or equal to this value the results become:

≤ 8.0 µg/mL	≥ 8.0 µg/mL
y-intercept = 0.430	y-intercept = 0.562
slope = 0.904	slope = 0.799
r = 0.917	r = 0.841

Thus, proportional error is lower and correlation is better at low doses.

FIGURE 21

SPLIT-SAMPLE ANALYSIS



y-intercept of about 1.0 µg/mL. An interesting situation arises if linear regression analysis is performed on all values less than 8.0 µg/mL and all those greater than or equal to 8.0 µg/mL. For values less than 8.0, the y-intercept becomes 0.430, the slope is 0.904, and r is 0.917, while at doses greater than 8.0 the values for these parameters are 0.562, 0.799, and 0.841, respectively. In other words, the proportional error is smaller and correlation is better at low as opposed to high doses. One explanation for this is that the precision and resolution of Pharmacia is poor at the higher doses as confirmed by the flat dose response curve and backbending of the Scatchard plot as found in this study. It is possible that the "insensitivity" of the Pharmacia kit is the problem, and that CN is actually a better procedure. The present evaluation has, in fact, indicated that the Pharmacia kit may be inferior.

Specificity

Concentrations of hGH up to 500 ng/mL and HCG up to 100 IU/mL were tested with the CN kit. Results are shown in figure 22. As can be seen, there was no significant cross reaction with either substance. This finding agrees well with that found by other researchers (94), and confirms the claims of the manufacturer. The B/B_0 values greater than maximum binding at 1:1 and 1:2 dilutions of cross-reacting substance were probably due to matrix effects produced

FIGURE 22

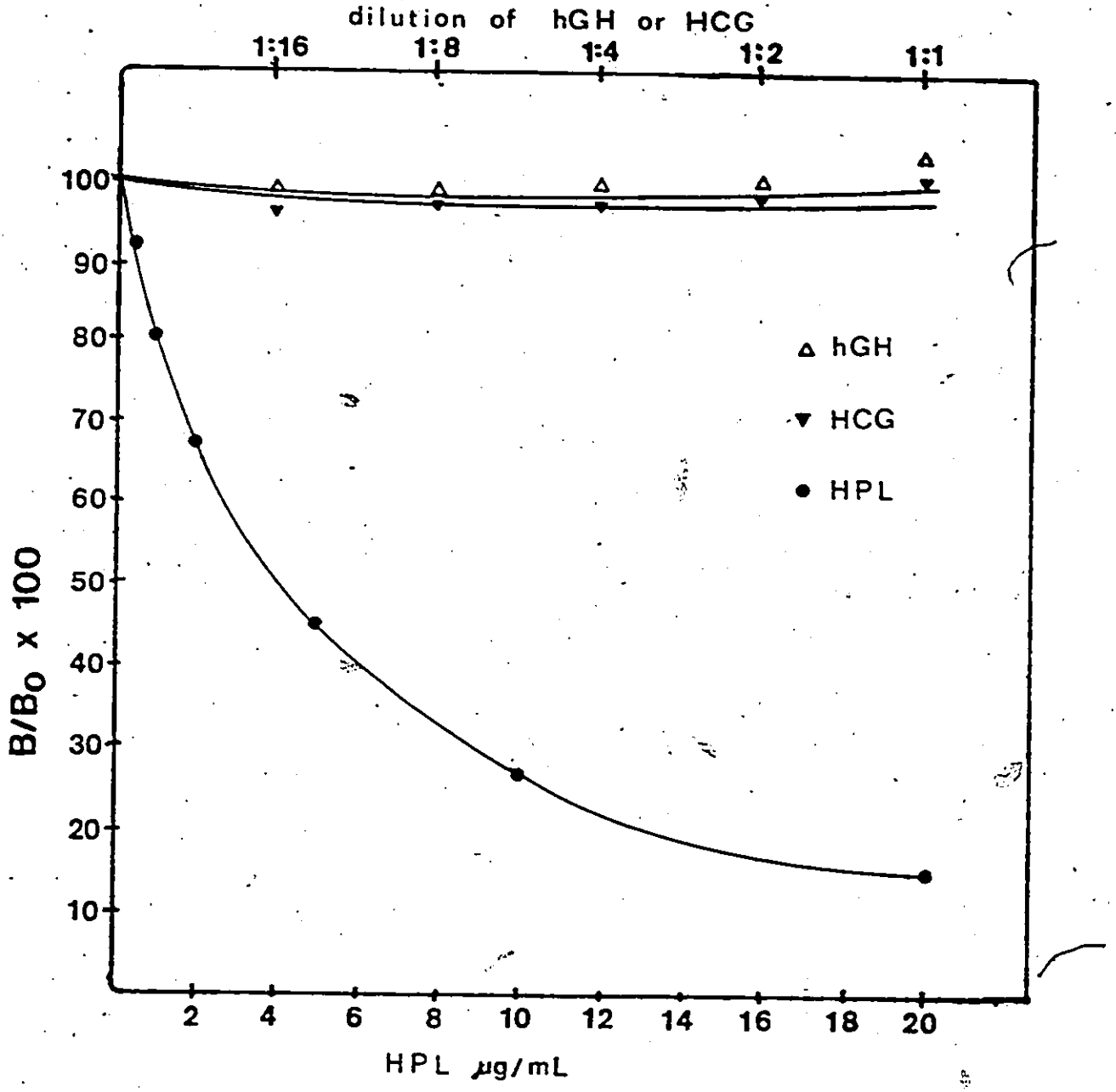
CROSS REACTION WITH hGH AND HCG

Legend

Initial (1:1) dilutions of hGH and HCG were comparable to concentrations of 500 ng/mL and 100 IU/mL in serum, respectively. No cross reaction was observed with either substance. A matrix affect may be influencing B/B_0 values at the 1:1 and 1:2 dilutions.

FIGURE 22

CROSS REACTION WITH hGH AND HCG



from the fact that the starting hGH and HCG solutions were aqueous, while dilutions were made up in increasing proportions of HPL-free serum.

Tracer Chromatography

To see if immunoreactivity of tracer was improved after purification by gel filtration, old tracer was run through a Sephadex G75 column. Fractions were collected to resolve components into damaged and undamaged peaks (Fig. 23). Three peaks were resolved, the fastest being Blue Dextran. Immunoreactivities performed on the fractions showed that the second peak, particularly the fractions in the center, had the highest immunoreactivity. This observation is similar to that found by Letchworth *et al.* in their development of an HPL procedure (90). There was essentially zero immunoreactivity of the third peak, and this fraction was assumed to be free iodide. Fractions 22 to 27, which composed the peak of the second component, were pooled. Immunoreactivity of this purified tracer was then compared to that of old tracer, and results are shown in Table VI. Purification greatly improved tracer immunoreactivity. If the amount of radioactivity present bound to Blue Dextran (assumed to be free ^{125}I) and that in fraction #3 are added, the percent of total radioactivity is about 37%. It is interesting to note that this corresponds well with the percent of radioactivity that is not bindable by excess antibody in the old

FIGURE 23

CHROMATOGRAPHY OF TRACE

Legend

Three radioactive fractions were resolved on Sephadex G75, the first being free ^{125}I bound to Blue Dextran, fraction #2 being immunoreactive labeled HPL, and fraction #3 being free ^{125}I.

FIGURE 23

CHROMATOGRAPHY OF TRACE

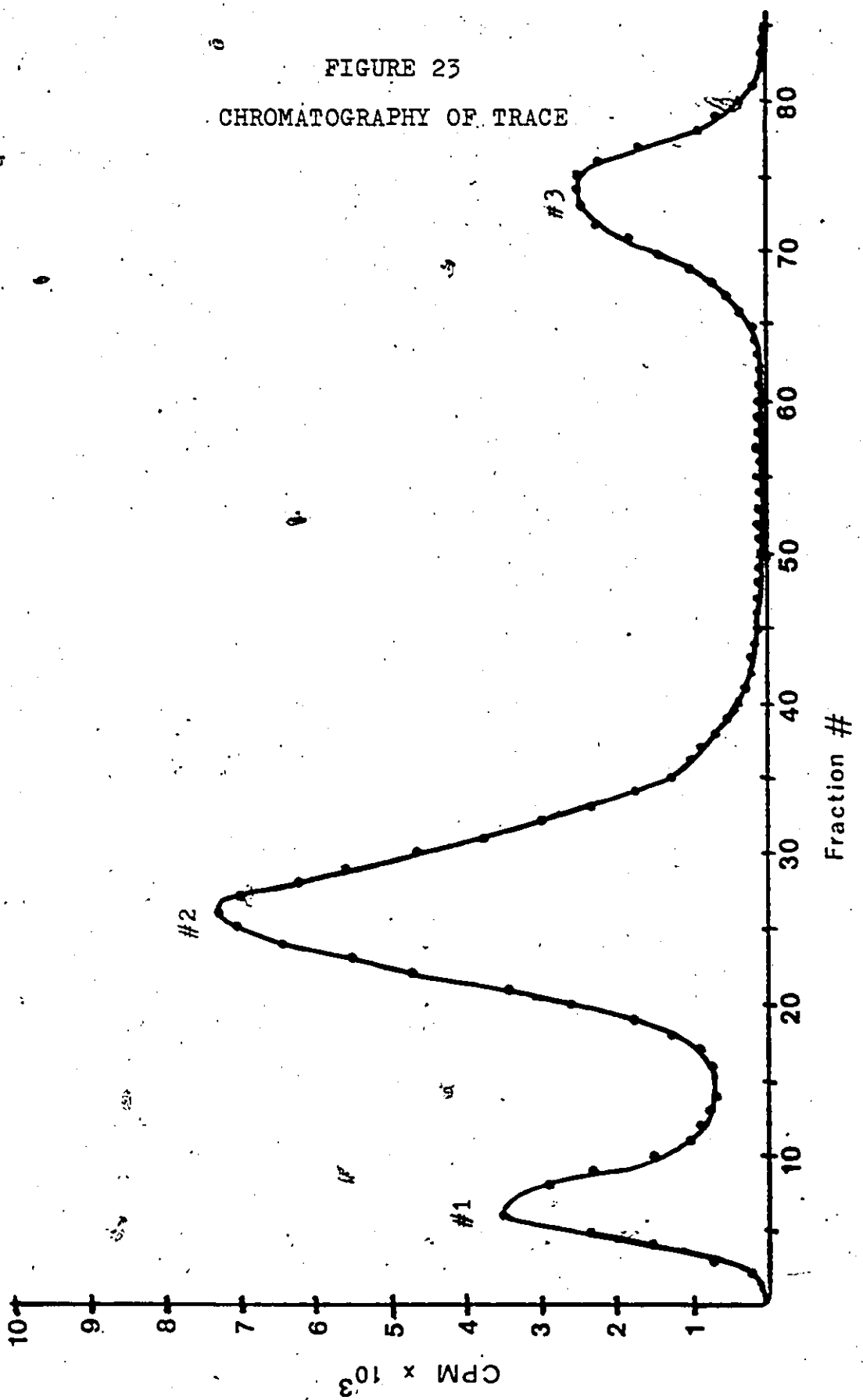


TABLE VI

IMMUNOREACTIVITY OF PURIFIED TRACE

Antibody concentration	% bound old tracer	% bound purified tracer
1x	49.2	77.3
5x	52.2	76.8
10x	53.8	77.2
20x	55.9	81.1
40x	57.2	81.1

tracer, i.e., about 43% (see Table VI).

Practical Considerations

A summary of practical and other miscellaneous considerations for kit evaluation is given in Table VII. Procedural and counting times were approximately equal for all three kits. Counting time had to be increased from 0.5 to 1.0 minute for P in order to obtain statistically significant counts (approximately 10,000) in the maximum binding tube. This resulted because of tracer age which caused both a low total count due to radionuclide decay and low maximum binding from damaged labeled ligand. Technically, A was the most simple procedure with easy reagent reconstitution and convenient sample and reagent volumes. The P kit required an inconvenient standard dilution (700 µL), while CN required both more complex reagent preparation and an inconvenient patient dilution step. Both A and P required separate standard curves and different antibody dilutions for samples from different stages of pregnancy, while with CN, all samples were run using only one curve and antibody dilution. Cost of reagents was least for P, but separating reagent (absolute ethanol) had to be supplied. Ethanol also had to be supplied for A--the most expensive of the procedures. The CN kit had an intermediate cost and no outside reagents had to be supplied.

Miscellaneous considerations included the fact that A

TABLE VII

PRACTICAL CONSIDERATIONS

	A	CN	P
Time:			
procedural:	30 min	30 min	30 min
counting:	0.5 min	0.5 min	0.5 to 1.0 min
Procedural ease:*	+++	+ (requires patient dilution)	++ (inconvenient std. dilution)
Price:	$1.17/tube (plus EtOH)	$0.95/tube	$0.66/tube (plus EtOH)
Miscellaneous:	3 different standard curves necessary	1 standard curve	2 different standard curves necessary
	only 4 stds. range up to 10 µg/mL	large range of stds.	
		controls provided with kit	

* number of +'s indicate increasing procedural ease

had only four standards and the range of the assay was only up to 10.5 µg/mL. Therefore, samples with concentrations above this value had to be diluted and repeated. CN had the greatest range (up to 20 µg/mL) and supplied the greatest number of standards. Two control specimens with second and third trimester values were also supplied with the kit.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Because of the backbending of the Scatchard plot, the high NSB, and the poor precision of P, it was concluded that this kit contained a very damaged tracer. After questioning the manufacturer, it was discovered that kits were not even shipped until approximately one month after labeling of tracer. Thus, kits received in the laboratory contained tracer about one month old, and were, therefore, usable for only two more weeks. Although parallelism studies appeared acceptable, and economically the kit was the most desirable, the kit was eliminated from further evaluation because of the above mentioned problems.

Scatchard plots for A and CN indiacted acceptable antisera. Precision was also within expected limits with both kits, and both showed good patient parallelism. The factors which came into play when choosing between these two kits were more practical considerations. Although reagent preparation was more complex, and a patient dilution was required in most cases with the CN kit, these factors were considered insignificant after more familiarization with the kit protocol. Also, since the sensitivity was greater and the range was larger, and because all samples could be assayed with one standard curve, CN was chosen for further evaluation. Other considerations which tended to favor use of CN were: the lower cost of the kit; the convenience of having all needed reagents

supplied in the kit (including control sera); and the fact that a double-antibody separation step is used--a theoretically better separation method. Finally, since the CN kit provided better linearization with the logit-log transformation, computer reduction of data could be used to speed up turn-around time. Amersham, on the other hand, did not adapt well to linear transformation on our computer, and for best results would have had to have been plotted manually.

Specificity of CN appeared to be acceptable but accuracy of results were in question. Recovery data indicated a positive proportional error, but results were inconclusive because adequate data was not obtained. Split-sample analysis done using specimens obtained from another laboratory also indicated a positive error; but, as mentioned earlier, the "reference" method was the Pharmacia kit--a kit indicated to be inferior by this evaluation. Since results obtained on the Ortho control sera agreed well with the target values of these controls, it was concluded that CN was accurate enough to put into routine use.

APPENDICES

APPENDIX A: TRACER SELF-DISPLACEMENT

Procedure:

1) Run normal standard curve.
2) Run tubes with no cold ligand and increasing amounts of tracer, i.e., 1x, 2x, 4x, 8x, etc.
3) Also determine NSB and total count for each tracer aliquot.

Calculations:

1) Correct each sample for its own NSB.
2) Calculate B/F for standards and increasing tracer aliquots.
3) Normalize each sample to the zero tube: $(B/F)/(B/F)_0$.
4) Plot $(B/F)/(B/F)_0$ vs. mass/tube for standard curve.
5) Read corresponding mass from curve of $(B/F)/(B/F)_0$ for tracer aliquots.
6) Divide each result by tracer aliquot to estimate 1x mass.
7) Calculate average mass (per tube): L_s
8) Calculate mass per volume of tracer solution: $L_s/V_t = \alpha$.

Reference: Midwest Radioassay Society Workshop; "RIA Kit Evaluation", Feb. 8, 1979, Henry Ford Hospital, Detroit, MI.

APPENDIX B:

SUMMARY GUIDE FOR CALCULATION OF COMPLETE SCATCHARD PLOT

Calculate mass contributed per tube by trace, $\mathbb{Q}$: **

$$\mathbb{Q} = \frac{T}{r \cdot E \cdot \sigma}$$

Calculate mass contributed per tube by quantity of standard cold ligand, D:

$$D = S_c \cdot V_s$$

Calcualte the total ligand mass in each reaction tube, L_t:

$$L_t = \mathbb{Q} + D$$

Calculate background corrected counts, C_c:

$$C_c = C_r - Bkg$$

Calculate complement of counts as bound or free counts as appropriate, C_b or C_f.

Calculate ratio of bound-to-total counts, C_b/T.

Calculate ligand bound, L_b:

$$L_b = L_t \, (C_b/T)$$

Calculate free ligand, L_f:

$$L_f = L_t - L_b$$

Calculate non-specific bound-to-free ratio, $(B/F)_N$:

$$(B/F)_N = \frac{Blk - Bkg}{T_c - Blk}$$

**This assumes that specific activity of the tracer is known (usually obtained from the manufacturer). If S.A. is not known, L_s determined from self-displacement may be stubstitued for $\mathbb{Q}$, or any amount up to 10% of the lowest standard may be arbitrarily substituted for this value.

Calculate non-specifically bound ligand, L_n:

$$L_n = L_f \cdot (B/F)_N$$

Calculate specifically bound ligand, B:

$$B = L_b - L_n$$

Calculate bound ligand per volume, mass bound:

$$\text{mass bound} = B/V_r$$

Calculate specific bound-to-free ligand ratio, B/F:

$$B/F = B/L_f$$

Combined general formulae:

y-axis:

$$B/F = \frac{\left(L_t \cdot \frac{C_b}{T}\right) - \left(L_f - (B/F)_N\right)}{L_t - \left(L_t \cdot \frac{C_b}{T}\right)}$$

x-axis:

$$\text{mass bound} = \frac{C_b}{T}(D + Q)$$

Reference: Midwest Radioassay Society Workshop: "RIA Kit Evaluation", Feb. 8, 1979, Henry Ford Hospital, Detroit, MI.

Symbols for Specific Activity And Scatchard Plot Calculations:

α	Tracer self-displacement estimate of ligand mass per volume (L_s/V_t)
B	Specific bound ligand
B/F	Ratio of specific bound ligand to free ligand (B/L_f)
$(B/F)_N$	Ratio of non-specific bound ligand to free ligand $\left(\frac{Blk - Bkg}{T_c - Blk}\right)$
$(B/F)_0$	Ratio of specific bound to free ligand in the maximum binding of zero standard tube $\left(\frac{B_0 - Blk}{T - B_0}\right)$
Blk	NSB tube, binding of tracer with no antibody present
Bkg	Counter background
C_b	Counts bound (equal to C_c unless C_c is free counts then $C_b = T - C_c$)
C_c	Background corrected raw counts (C_r - Bkg)
C_f	Counts free (equal to C_c unless C_c is bound counts then $C_f = T - C_c$)
C_b/T	Fraction of counts (and presumably total ligand) bound. Also indicated as B/T
D	Ligand mass contributed to reaction tube by standard ($S_c \times V_s$)
L_b	Total ligand bound ($L_t \times C_b/T$) (mass)
L_f	Free ligand ($L_t - L_b$) (mass)
L_n	Non-specific bound ligand ($L_f \times (B/F)_N$) (mass)
L_s	Tracer self-displacement estimate of ligand mass
L_t	Total ligand concentration in reaction tube (D + Q) (mass)

r	Conversion constant equal to 2.2×10^6 DPM/μCi
S_c	Standard concentration
T_c	Raw total counts
T	Background corrected total counts
V_r	Reaction volume
V_s	Standard volume
V_t	Tracer volume
E	Counter efficienty (CPM/DPM)
σ	Tracer specific activity (uCi/mass)
Q	Ligand dose in reaction tube contributed by tracer (mass)

APPENDIX C:

STATISTICS COMMONLY USED IN METHOD COMPARISONS

I. Linear Regression:

BASIC WORKSHEET FOR ALL TYPES OF LINEAR RELATIONSHIPS

X denotes _____ Y denotes _____

ΣX = _____ ΣY = _____

$\bar{X}$ = _____ $\bar{Y}$ = _____

Number of points: n = _____

Step (1) ΣXY = _____

(2) $(\Sigma X)(\Sigma Y)/n$ = _____

(3) S_{xy} = Step (1) − Step (2)

(4) ΣX^2 = _____ (7) ΣY^2 = _____

(5) $(\Sigma X)^2/n$ = _____ (8) $(\Sigma Y)^2/n$ = _____

(6) S_{xx} = Step (4) − Step (5) (9) S_{yy} = Step (7) − Step (8)

(10) $b_1 = \frac{S_{xy}}{S_{xx}}$ = Step (3) ÷ Step (6) (14) $\frac{(S_{xy})^2}{S_{xx}}$ = _____

(11) $\bar{Y}$ = _____ (15) $(n-2)s_{y\cdot}^2$ = Step (9) − Step (14)

(12) $b_1\bar{X}$ = _____ (16) $s_{y\cdot}^2$ = Step (15) ÷ $(n-2)$

(13) $b_0 = \bar{Y} - b_1\bar{X}$ = Step (11) − Step (12) $s_{y\cdot}$ = _____

Equation of the line:

$Y = b_0 + b_1X$

_____

s_{b_1} = _____

s_{b_0} = _____

Estimated variance of the slope:

$s_{b_1}^2 = \frac{s_{y\cdot}^2}{S_{xx}}$ = Step (16) ÷ Step (6)

Estimated variance of intercept:

$s_{b_0}^2 = s_{y\cdot}^2\left(\frac{1}{n} + \frac{\bar{X}^2}{S_{xx}}\right)$ = _____

Note: The following are algebraically identical:

$S_{xx} = \Sigma(X - \bar{X})^2$; $S_{yy} = \Sigma(Y - \bar{Y})^2$; $S_{xy} = \Sigma(X - \bar{X})(Y - \bar{Y})$.

Reference: Natrella, M.G.: Experimental Statistics: National Bureau of Standards Handbook 91, U.S. Government Printing Office, Washington. (1966), p. 5-10

II. Paired t-Test:

reference method = X test method = Y

n = number of observations

Calculate:

1) $\overline{X} = \frac{\Sigma X}{n}$

2) $\overline{Y} = \frac{\Sigma Y}{n}$

3) bias = $\overline{X} - \overline{Y}$

4) difference between X and Y: $X_i - Y_i = D$ (for each pair)

5) difference minus bias: D - bias (for each i)

6) square of step 5: $(D - \text{bias})^2$ (for each i)

7) sum of squares: $\Sigma(D - \text{bias})^2$

8) standard deviation of difference: $\sqrt{\frac{\Sigma(D - \text{bias})^2}{n - 1}} = SD_d$

9) $t = \frac{\text{bias} \times \sqrt{n}}{SD_d}$

Compare to t-value from t-tables at desired significance level.

Reference: Barnett, M.D. Youden, W.J.: "A Revised Scheme for The Comparison of Quantitative Methods", Amer. J. Clin. Path, 54, (1970) Suppl., p. 456.

III. Correlation Coefficient Calculation:

Compute: (see APPENDIX C p. 151)

ΣY

ΣY^2

ΣX

ΣX^2

ΣXY

$$S_{xx} = X^2 - \frac{(\Sigma X)^2}{n}$$

$$S_{yy} = Y^2 - \frac{(\Sigma Y)^2}{n}$$

$$S_{xy} = \Sigma XY - \left((\Sigma X)(\Sigma Y)\right)/n$$

Correlation coefficient:

$$r_{xy} = \frac{S_{xy}}{\sqrt{S_{xx}\, S_{yy}}}$$

Reference: Sokal, R.R., Rohlf, F.J.: Biometry, W.H. Freeman and Co., San Francisco, (1969), p. 509.

APPENDIX D: COMPUTER CALCULATIONS

I. Standards

$T = \overline{T}$ = mean of total count tubes in CPM

$$\text{Pipetting error} = \frac{(S.D.)_T^2 - \overline{T}}{\overline{T}} \times 100$$

$(S.D.)_T$ = standard deviation of total counts

$(S.D.)_T^2$ = variance of total counts

$N = \overline{N}$ = mean of non-specific binding or blank tubes

$B\emptyset = \overline{B\emptyset} - \overline{N}$ = mean counts for zero standard corrected for NSB

$B\emptyset/T = \frac{\overline{B\emptyset} - \overline{N}}{\overline{T} - \overline{N}} \times 100$ = initial percent bound corrected for NSB

$B = \overline{B} - \overline{N}$ = bound counts for any tube corrected for NSB

$B/B\emptyset = (\overline{B} - \overline{N})/(\overline{B\emptyset} - \overline{N})$ = percent bound normalized to zero tube and corrected for NSB

$$S.D. = \text{standard deviation} = \frac{\Sigma y_i^2 - \frac{(\Sigma y_i)^2}{n}}{n - 1}$$

where $y_i = B/B\emptyset$

$$\text{Coefficient of variation} = \frac{S.D.}{\text{mean}} \times 100$$

where mean $= (\Sigma y_i)/n$

n = number of replicates

II. Regression Calculations:

for Rodbard (logit-log plot):

$\text{logit } y = a + b \log_{10} x$ where y = dose response variable

x = dose

and logit $y = \ln \frac{B/B\emptyset}{1-(B/B\emptyset)}$

$$\text{Slope} = b = \frac{\Sigma x_i y_i - \frac{\Sigma x_i \, \Sigma y_i}{n}}{\Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}}$$

Y intercept = $a = \bar{y} - b(\bar{x})$

(Note: This is y-crossing where $\log_{10} x = 0$; i.e., at dose concentration equal to 1 for the Rodbard model.)

EDk = estimated dose in concentration units at k% binding

$$= \frac{\text{logit } \frac{k}{100} - a}{b}$$

r^2 = coefficient of determination = $\frac{\left(\Sigma x_i y_i - \frac{\Sigma x_i \, \Sigma y_i}{n}\right)^2}{\left(\Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}\right)\left(\Sigma y_i^2 - \frac{(\Sigma y_i)^2}{n}\right)}$

(for unweighted regressions only)

Iterated Weighted Regression:

$$\text{Weight} = W = \frac{1}{\text{variance}}$$

where variance = $(\text{S.D.})^2$

variance is determined for each dose x and is used to weight each individual point.

W_1 = sum of weights for all points of a single iteration

$$\text{Slope} = b = \frac{\Sigma W x_i y_i - \frac{(\Sigma W x_i)(\Sigma W y_i)}{W_1}}{\Sigma W x_i^2 - \frac{(\Sigma W x_i)^2}{W_1}}$$

Y intercept = $y - b(x)$

$$x = \frac{\Sigma W x_i}{\Sigma W} \qquad y = \frac{\Sigma W y_i}{\Sigma W}$$

Steps to solution:

1. Calculate $\hat{y}$ for each dose x using regression coefficients form previous iteration. For the first iteration use coefficients calculated for the unweighted regression.

$$\text{logit } y = a_i + b_i \log_{10} x$$

2. Calculate a new weight for each dose using:

$\frac{\Sigma (y_i - \hat{y})^2}{n - 1}$ = variance, where $\hat{y}$ is the response evaluated for each x from the previous iteration.

3. Run regression incorporating weights.

4. Repeat steps 1 through 3 until 5 iterations are completed.

5. Print coefficients.

$$\text{Calculated dose} = 10^{\left(\frac{\text{logit } y - a}{b}\right)}$$

$$\text{Difference} = \frac{\text{actual dose} - \text{calculated dose}}{\text{actual dose}} \times 100$$

III. Controls And Unknowns:

$B = \overline{B} - \overline{N}$ = bound counts corrected for NSB

$B/B\emptyset = (\overline{B} - \overline{N})/(\overline{B\emptyset} - \overline{N})$ - percent bound normalized to zero tube and corrected for NSB.

Calculated dose = C.D. =

$$\frac{10^{\left(\frac{\text{logit } y - a}{b}\right)} \times \text{aliquot factor}}{\% \text{ recovery}/100\%}$$

aliquot factor = dilution of patient

% recovery used only for extraction procedures, if no estraction, recovery = 100%.

95% range = C.D. of (mean + 2 x S.D.)
C.D. of (mean − 2 x S.D.)

where mean = $\frac{x_i}{n}$ $\quad x_i$ = CPM

S.D. = standard deviation = $\frac{\Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}}{n-1}$

C.V. = coefficient of variation = $\frac{\text{S.D.}}{\text{mean}} \times 100$

Reference: "Hewlett Packard 9831 Radioimmunoassay #09831-14254 Software Information", Hewlett Packard, Loveland. Co.

IV. A Sample Computer Printout:

STANDARDS:

	POSITION #	CPM-NSB			
TOTALS	1	91610			
	2	91158			
	AVG.=	91384	PIPETTING ERROR	0.2%	
NSB'S	3	6312			
	4	6272			
	AVG.=	6292			
B0'S	5	65803			
	6	67308			
			B0/T	B/B0	S.D.	COEF OF VAR
	B0=	66558	72.8%	1.000	0.016	1.6%

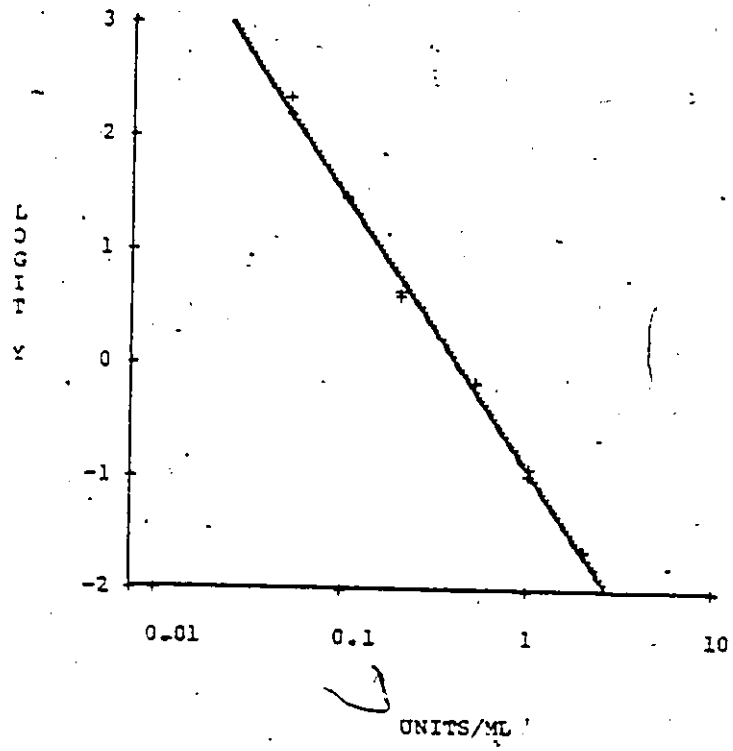
DOSE	POSITION #	CPM-NSB	B/B0	S.D.	COEF OF VAR
0.05	7	59922			
	8	60752			
	B=	60337	0.907	0.009	1.0%
0.10	9	54022			
	10	54108			
	B=	54065	0.812	0.001	0.1%
0.20	11	43480			
	12	42942			
	B=	43211	0.649	0.006	0.9%
0.50	13	30456			
	14	30604			
	B=	30530	0.459	0.002	0.3%
1.00	15	18158			
	16	19056			
	B=	18607	0.280	0.010	3.4%
2.00	17	10780			
	18	10800			
	B=	10790	0.162	0.000	0.1%

*=TUBE DELETED
**=HIGH VARIATION

UNWEIGHTED ROBBARD MODEL

SLOPE=	-2.415
Y INTERCEPT=	-0.941
ED20=	1.529
ED50=	0.408
ED80=	0.109
r^2=	0.998

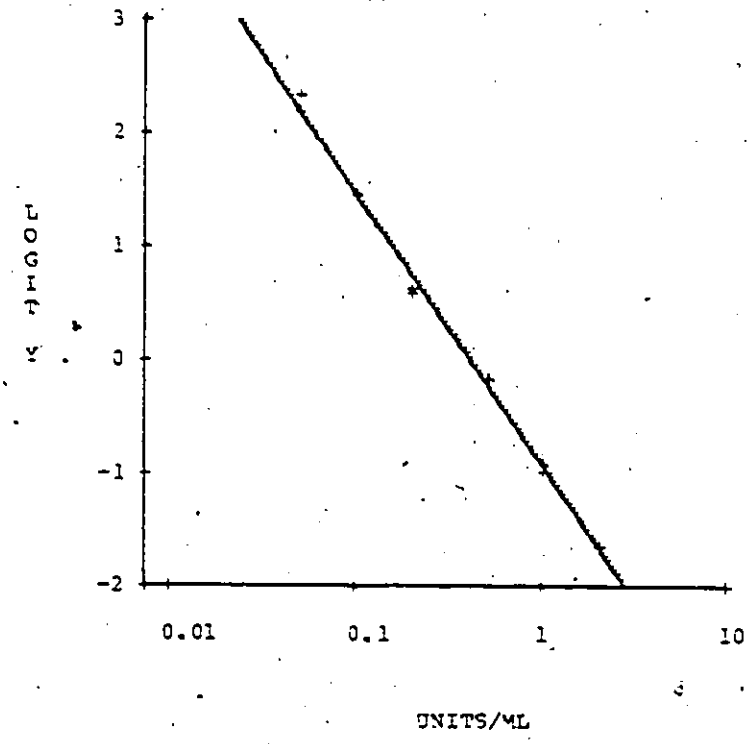
ACTUAL DOSE	CALCULATED DOSE	DIFFERENCE
0.05	0.05	5.6%
0.10	0.10	-0.8%
0.20	0.23	-13.3%
0.50	0.48	4.5%
1.00	1.01	-0.5%
2.00	1.95	2.4%



WEIGHTED RODBARD MODEL

SLOPE=	-2.389
Y INTERCEPT=	-0.924
ED20=	1.562
ED50=	0.411
ED80=	0.109

ACTUAL DOSE	CALCULATED DOSE	DIFFERENCE
0.05	0.05	8.13
0.10	0.10	-0.09
0.20	0.23	-13.49
0.50	0.48	3.79
1.00	1.02	-2.38
2.00	2.00	0.09



CONTROLS:

ALIQUOT FACTOR= 10.000

CONTROL #	POSITION #	CPM	B/B0	UNWEIGHTED WEIGHTED RODBARD	RODBARD	FERNANDEZ	COEF OF VAR
1	19	20291					
	20	20009					
	B=	34008	0.511	3.91	3.94	3.81	1.2
2	21	13661					
	22	13379					
	B=	20748	0.312	8.68	8.81	8.63	1.9

*=TUBE DELETED
**=HIGH VARIATION
***=INVALID EFFICIENCY

UNKNOWNS: UNWEIGHTED RODBARD MODEL.

ALIQUOT FACTOR= 10.000

PATIENT #	POSITION #	CPM	B/B0	CALCULATED DOSE	95% RANGE	COEF OF VAR
1	23	13022				
	24	12874				
	B=	19604	0.295	9.38	9.11 9.65	1.1%
2	25	12811				
	26	12858				
	B=	19377	0.291	9.52	9.44 9.61	0.3%
3	27	11949				
	28	12397				
	B=	18054	0.271	10.46	9.56 11.49	3.5%
4	29	11114				
	30	11159				
	B=	15981	0.240	12.23	12.11 12.35	0.4%
5	31	10859				
	32	10468				
	B=	15035	0.226	13.19	12.08 14.48	3.7%
6	33	11330				
	34	11262				
	B=	16300	0.245	11.93	11.75 12.11	0.6%
7	35	10187				
	36	10081				
	B=	13976	0.210	14.42	14.06 14.81	1.1%
8	37	10755				
	38	10740				
	B=	15213	0.229	13.00	12.93 13.08	0.2%
9	39	10828				
	40	10737				
	B=	15323	0.230	12.89	12.77 13.01	0.4%
10	41	10831				
	42	11009				
	B=	15548	0.234	12.66	12.16 13.18	1.6%
11	43	11900				
	44	11924				
	B=	17532	0.263	10.87	10.81 10.92	0.2%
12	45	11350				
	46	11651				
	B=	16709	0.251	11.56	10.85 12.35	2.5%

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